

BONE MATRIX AND BONE FORMATION

An experimental study of
demineralized bone
reimplanted in bone defects

Jan Wittbjer

Lund 1983



Malmö 1983

BONE MATRIX AND BONE FORMATION

An experimental study of demineralized bone
reimplanted in bone defects.

AKADEMISK AVHANDLING

som för avläggande av odontologie doktorsexamen
vid odontologiska fakulteten, Lunds universitet,
offentligen kommer att försvaras i aulan,
Tandläkarhögskolan, Malmö
onsdagen den 11 maj 1983, kl 09.00

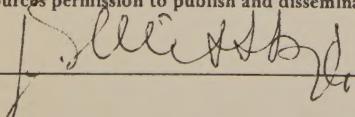
av

Jan Wittbjer
leg tandläkare

Organization LUND UNIVERSITY Department of Prosthetic Dentistry Faculty of Odontology University of Lund Malmö, Sweden	Document name DOCTORAL DISSERTATION Date of issue May 1983 CODEN: LUODD5/(ODPR-1003)/1-120/(1983)
Author(s) Jan Wittbjör	Sponsoring organization
Title and subtitle BONE MATRIX AND BONE FORMATION An experimental study of demineralized bone reimplanted in bone defects	
Abstract The bone formation in a segmental bone defect grafted with demineralized autologous bone was studied in rabbits. The progress of mineralization was estimated by light- and fluorescence microscopy, roentgenology including planimetry and scintigraphy. Of these methods the scintigraphic technique proved to be of particular value as it reveals exclusively new bone formation, when demineralized bone is grafted. With the evaluation methods used it was concluded that demineralization of compact autologous bone gives a better grafting material than undemineralized compact bone. The addition of fresh autologous marrow hastens the incorporation of the demineralized graft and accentuates its osteogenicity during the first few postsurgical weeks. After four weeks no difference was, however, observed in bone forming activity between grafts with and without additional marrow. Locally administered biosynthetic human growth hormone was not shown to have any effect on the osteogenetic activity. Rather a delay was noticed probably due to a dilution of biochemical components involved in the induction process. Direct electric currents were shown to interfere negatively with the osteogenetic activity during the initial 14 days. Increased bone forming activity was however noticed from 14 to 28 days postoperatively indicating a possible usage of electric currents in later phases of the bone forming process.	
Key words bone transplant, demineralized bone, electric currents, growth hormone, scintigraphy	
Classification system and/or index terms (if any)	
Supplementary bibliographical information	Language English
ISSN and key title	ISBN 91-7222-594-7
Recipient's notes	Number of pages 120 Security classification
Price	
Distribution by (name and address)	

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature



Date 15 april 1983

BONE MATRIX AND BONE FORMATION

An experimental study of
demineralized bone
reimplanted in bone defects

Jan Wittbjer

From the Departments of Prosthetic Dentistry and Oral Radiology, Faculty of Odontology,
Plastic Surgery, Malmö General Hospital and Orthopaedics, University Hospital Lund,
University of Lund, Sweden

Malmö 1983

The English regarding the summary has been revised
by Mr. Peter Baston, English Centre, Malmö, Sweden.



Printed in Sweden by Infotryck ab, Malmö, 1983.
ISBN 91-7222-594-7

To Kerstin and my parents

CONTENTS	PAGE
PREFACE	6
INTRODUCTION	7
AIMS	10
MATERIAL AND METHODS	11
RESULTS	18
GENERAL DISCUSSION	23
GENERAL SUMMARY	35
ACKNOWLEDGEMENTS	37
REFERENCES	38
PAPER I	45
PAPER II	51
PAPER III	57
PAPER IV	67
PAPER V	93

PREFACE

The present thesis is based on the following publications, which will be referred to by their Roman numerals.

- I. Wittbjer, J., Palmer, B. & Thorngren, K-G.

Osteogenetic properties of reimplanted decalcified and undecalcified autologous bone in the rabbit radius.

Scand. J. Plast. Reconstr. Surg. 1982, 16, 239-244.

- II. Wittbjer, J., Nosslin, B., Palmer, B. & Thorngren, K-G.

Bone formation of transplanted autologous bone matrix in rabbit evaluated by technetium radionuclide bone imaging.

Scand. J. Plast. Reconstr. Surg. 1982, 16, 23-28.

- III. Wittbjer, J., Palmer, B., Rohlin, M. & Thorngren, K-G.

Osteogenetic activity in composite grafts of demineralized compact bone and marrow.

Clin. Orthop. 1983, 173, 255-264.

- IV. Wittbjer, J., Rohlin, M. & Thorngren, K-G.

Bone formation in demineralized bone transplants treated with biosynthetic human growth hormone.

Scand. J. Plast. Reconstr. Surg. (In press)

- V. Wittbjer, J., Glantz, P-O., Rohlin, M. & Thorngren, K-G.

On direct currents and bone formation in demineralized bone transplants.

Acta Odontol. Scand. (In press)

INTRODUCTION

Facial disability, acquired or reflecting a congenital condition, is generally traumatic for the individual and leads to problems in psychosocial adjustment. As major changes in facial morphology are often also combined with difficulties in providing nourishment, there is an obvious need to treat and restore tissues of the stomatognathic system and its associated craniofacial structures. Extensive experimental and clinical studies have been performed in an effort to solve this problem which is often associated with the need to provide sufficient amounts of soft and hard tissues in combination or separately. After excision of hard tissue the resultant interruption in continuity raises bone grafting problems not least finding suitable bone in sufficient amounts. This is also the case in bone defects in other parts of the body. Though fresh autogeneic bone is the grafting material preferred many substitutes have been tried with varying degrees of success. Already in 1889 Senn used demineralized bone grafts to fill cavities after excision as a treatment of osteomyelitis. The results were, however, conflicting and not until the last two decades were truly successful attempts made to find substitutes for fresh autogeneic grafts by using various preparations of bone (Williams 1964, Emmings et al. 1966, Gage et al. 1966, Marcove & Miller 1969, Smith & Weaver 1976, Urist 1980, Friedlaender 1982).

The clinical success in man with preserved allografts has, however, not been unambiguous, probably due to the antigenicity of the grafts (Burchardt et al. 1977, Musculo &

Kawai 1977, Musculo, Kawai & Roy 1977) which sooner or later will give rise to resorption. Attempts have therefore been made to reduce the level of antigenicity (Marciani et al. 1967, Parrish 1973, Osborne et al. 1977, Urist & Dawson 1981). Presuming this rejection mechanism is a major cause for failures, it is likely that autogeneic bone would be preferable when used in a devitalized and demineralized form as a substitute for fresh autogeneic bone. Due to the importance of restoring form and function it is therefore probable that the reuse of the patients' own resected bone, could be an alternative in reconstruction as it fits perfectly, and lacks antigenous properties and does not require any additional surgical procedures in order to retrieve a grafting material. To obtain favourable results the principles for bone formation in grafted demineralized autogeneic bone have, however, to be considered.

As in fracture healing, the incorporation of a bone graft depends on the proliferative elements in the periosteum, the endosteum, the bone marrow and the existing osteogenetic cells of the microenvironment. This phenomenon, known as osteoconduction, explains that incorporation also occurs within a framework of such a non-biologic group of material as ceramics and corall or in non-viable biologic material such as autoclaved, deproteinized, chemosterilized, frozen or freeze-dried bone (Urist, 1980).

Another mechanism for bone formation, osteoinduction, holds that mesenchymal cells proliferate and differentiate to cells

with osteogenetic competence in a type of substrate - cell interaction of bone matrix and adjacent connective tissue cells (van de Putte and Urist 1965, Urist 1970). It would then be evident that osteoconduction and osteoinduction are inter-related processes giving a rapid and complete incorporation of bone grafts and healing of fractures. The osteoconductive and osteoinductive phases are thus closely associated processes progressing towards incorporation of the donor tissue to the recipient site. Induction is a property found not only in living tissues but also in devitalized ones or in tissue extracts (Holtfreter 1933). This observation has been confirmed by Urist, Mikulski and Lietze (1979); Mizutani & Urist (1982), who also found inductive capacity in bone matrix extract. This non-collagenous hydrophobic glycoprotein is called bone morphogenetic protein (BMP).

This experimental series was undertaken with the intention of investigating the prerequisites for bone formation after resection, when reusing an excised demineralized piece of bone as a bone graft. The bone forming capacity of the implants were tested in a clinically applicable experimental model and with evaluation methods which made it possible to determine the speed of bone formation, thus comparing different methods of enhancing bone formation.

AIMS

The aims of the present investigation were to:

- a. develop a method for determining bone forming activity under various experimental conditions.
- b. compare the osteogenetic properties in demineralized non-vital autogeneic bone with those of undemineralized non-vital autogeneic bone transplanted into bone defects.
- c. compare the healing of bone defects grafted with demineralized non-vital autogeneic bone with that of contralateral defects left without graft.
- d. evaluate the effects on the healing of bone defects by a combination of fresh autogeneic bone marrow and demineralized non-vital autogeneic bone.
- e. study the effects of growth hormone on the healing of bone defects grafted with demineralized non-vital autogeneic bone.
- f. study the effects of electric currents on the healing of bone defects grafted with demineralized non-vital autogeneic bone.

MATERIAL AND METHODS

A General outline

Adult rabbits with an average body weight of 3.6 kilograms were used for the experiments. The rabbits were operated on under anaesthesia consisting of intravenous injection of Mebumal vet.[®] (LEO, Helsingborg, Sweden), supplemented with local anaesthesia (Xylocain[®] 0,5 %, Astra, Södertälje, Sweden).

A piece of radius including periosteum was sawn off bilaterally during continuous irrigation with ample amounts of saline. A piece of sterile plastic tubing (Nelaton[®] catheter, Steritex, Denmark) with dimensions corresponding to those of the resected pieces of bone was placed in each radial defect and the wounds were closed in anatomical planes. The two pieces of bone were then cleaned from periosteum and marrow and treated in different predetermined ways according to the current experiment using one side as the test side and the contralateral as a control side. At a second operation three days later the wounds were bilaterally opened, the tubes were removed and the bone grafts were replaced in the radial defects.

B Experiments

1 Demineralization (I-V):

Some of the resected pieces of bone were demineralized in 0.6 N HCl for 24 hours and then repeatedly rinsed in saline until the storage solution reached pH 6.5. These bone pieces were then kept in saline at +4⁰ C for the following two days.

The demineralization was checked radiographically.

2 Comparisons between the influence of demineralized and undemineralized bone on the healing of bone defects (I):

In order to compare the osteogenetic properties of demineralized and undemineralized autogenetic bone grafted to the radial bone defect, one resected radius bone was demineralized in 0.6 N HCl and the other only rinsed from periosteum and marrow. Three days after the first operation the pieces of bone were grafted to the radial defect and left in place for 28 days. The healing process was examined during the running experiment by roentgenography. After the animals were sacrificed the dissected specimens were analyzed by roentgenography as well as by light- and fluorescence microscopy.

3 Comparisons between the healing of bone defects with and without grafted demineralized bone (II):

The healing of radial defects where demineralized autogeneic grafts had been used was compared with that of corresponding defects left without grafts. The demineralized piece of bone not used was placed in a skin tube on the back of the rabbit. The skin tube was made by mobilizing a skin flap including Musculus panniculus carnosus in which a pouch was created where the demineralized bone was positioned. This investigation lasted for 6 months and the bone forming process was studied by roentgenography and scintigraphy three and six months after operation, respectively.

4 Influence of composite grafts (III):

The contribution of fresh autologous bone marrow to the healing of bone defects which had been grafted with demineralized autogeneic bone, was studied by comparing the difference in bone forming properties between demineralized bone with and without supplemented marrow. The bone marrow was obtained by aspiration from the femoral medullary cavity and immediately administered into the defect. The bone formation was evaluated by roentgenography including planimetry, scintigraphy and autoradiography at 14 and 28 days after operation.

5 Influence of growth hormone (IV):

The influence of growth hormone was studied by distributing biosynthetic human growth hormone produced by hybrid DNA-technique (Kabi, Solna, Sweden) to rabbit bone defects which had been grafted with demineralized autogeneic bone. The growth hormone was administered by an osmotic pump (Alzet[®], Model 2 ML2, ALZA Corporation, Palo Alto, U.S.) during the first 14 days postoperatively. The bone forming process on the experimental side was compared with that on the contralateral side which had been treated in the same way except saline had been given instead of growth hormone. The bone forming process was evaluated by scintigraphy and roentgenography including planimetry at 14 and 28 days after operation. From freeze-sectioned specimens autoradiography was also performed 28 days after operation.

6 Influence of direct currents (V):

The influence of direct currents on the mineralization of bone defects grafted with demineralized autogeneic bone was studied in the following way. Two platinum wires were encircled around demineralized pieces of bone which had been implanted in the defects. One wire was connected to a power source delivering 20 μ A constant current during the experimental period. This wire was connected to serve as a cathode. The second wire was left disconnected and used as a control. The anodes were located over the muscle fascia at an approximate distance of 40 mm from the defects. The influence on bone formation was evaluated according to the same methods as described for the growth hormone study.

C Evaluation methods

The bone forming processes were evaluated by the following methods: light- and fluorescence microscopy (I), roentgenographic examinations (I, II, III, IV, V) including planimetry (III, IV, V), scintigraphy (II, III, IV, V) and autoradiography (III, IV, V).

1 Light- and fluorescence microscopy (I):

After the rabbits were sacrificed their forelegs were amputated 10 mm distal and proximal to the bone defects. Soft tissues were removed and roentgenograms of the specimens were taken. Guided by these roentgenograms the specimens were then sectioned in two pieces of equal size. One half was put in formaline (10 %), then demineralized and serially sectioned and finally stained with haematoxylin and eosin.

The other half was fixed in absolute alcohol, embedded in plastic and cut transversely with a grinding disc. In light microscopy bone formation and bone resorption was evaluated in the haematoxylineosin sections by measuring the areas of newly formed bone and of resorption in the grafted bone. Morphometrical evaluations of active bone formation in the plastic embedded sections were performed in fluorescence microscopy by using a grid according to the method described by Merz and Schenk (1970). The percentage volumetric density of OTC (oxytetracycline) deposition was thus determined.

2 Roentgenography (I-V)

During the course of the experiments the forelegs of the animals were examined with a dental X-ray unit (Oralix 65, N.V. Philips, Eindhoven, Netherlands) using Kodac Occlusal Ultra-Speed D films and an exposure time of 0.4 second. These radiographic examinations were performed at predetermined intervals.

In studies III-V the roentgenograms of the forelegs were placed in an X-ray film enlarger/viewer (Realist Inc. Photographic Division, Menomonee Falls, Wisconsin) having an optic magnification of 10:1. The display was covered with transparent paper on which the outlines of the mineralized tissues in the bone defects were drawn. The extent of mineralization was then estimated with a planimeter (Los Angeles Scientific Instruments Co., Inc.). The results were expressed as the percentage of mineralized area of the total bone defect area.

3 Scintigraphy (II-V)

For scintigraphy $^{99}\text{Tc}^{\text{m}}$ -labelled MDP (methylene-diphosphonate) was used as evaluation method in studies II and III and $^{99}\text{Tc}^{\text{m}}$ -labelled DPD (dicarboxypropane diphosphonate) in studies IV and V. The radioactive substance was given intravenously to the rabbits via an ear vein three hours before the scintigraphic investigation. At the actual examination the anaesthetized rabbits or specimens were placed on a mobile table. A gamma camera with pin-hole collimator having an opening of 6 mm and a length of 35 cm was focused on the foreleg at a distance of 65 mm between the upper surface and the collimator opening. In the computer display this distance gave a picture of the radius, the ulna and the distal part of the humerus. The emitted gamma radiation was recorded in the camera which was connected to a computer. Given numerical data were stored in a disc memory.

To evaluate and make possible to calculate the relative values of activity in the defects, regions of interest were mapped on the computer-visualized picture. A control area including 300 pixels (picture-elements) was chosen at a distance of 10 mm from the resection end to avoid influence from the activity of the callus formation. From this control area about 10 more consecutive areas were chosen in the proximal direction. Mean counts were measured separately from every region of interest and the differences from the control area were calculated. Relative values in relation to the control area were

then calculated for each region.

4 Autoradiography (III-V)

Immediately after the scintigraphic examinations, the forelegs from randomly chosen rabbits were freeze-sectioned in a microtome. The sections, 20 μm thick, were kept under refrigeration (-15°C) and autoradiographic examinations were performed by apposition of the sections against films type Structurix D7 (Agfa-Gevaert, Antwerpen, Belgium). The exposure time used was approximately 72 hours. After exposures the sections were separated from the films and freezedried. The films were developed with a developer, type Gevaert G 334, for 10 minutes.

RESULTS

1 Experiments with undemineralized bone graft (I)

Seven days after implantation the roentgenograms showed a periosteal callus formation at the sawn ends but no changes within the grafts (Fig. I: 1). After 28 days no substantial additional changes could be noticed (Fig. I: 4). The histologic examinations revealed small islands of newly formed bone in resorption lacunae within limited areas in the periphery (Fig. I: 7). The morphometric analysis showed only small areas of newly formed bone (Table I: 1).

2 Experiments with ungrafted bone defect (II)

Three months after operation a narrowing callus formation was seen at the resection ends, in some cases reaching almost into the central part of the defect. In all defects but two the amount of bone formed was not considered as a bridging mineralization and the bone forming activity shown in the scintigraphic registration was almost as low as at the control area outside the defect. Over the period of six months no further healing tendency was noticed in the ungrafted defects (Fig. II: 5). The scintigraphic registration showed no appreciable activity at the end of the experimental period (Fig. II: 2 B).

3 Experiments with scin tubes (II)

Three months after implantation of the demineralized bone a radiopacity corresponding to the implant was

noticed in the skin tubes. After six months a slight additional increase in radiopacity was noticed (Fig. II: 4).

4 Experiments with demineralized bone graft (I-III)

Fourteen days after implantation the roentgenograms showed reactive callus formation at the sites of resection and levels of radiopacity indicating mineralization partly filling the defects (Fig. III: 2 C). The scintigrams showed two peaks representing the resection ends, and relative low activity in the centre of the defect, all corresponding with the findings in the radiographs (Figs. III: 1 A and 2 C; Table III: 2) and the autoradiographs (Fig. III: 2 D). In addition the planimetric estimation indicated mineral deposition occupying almost half of the initial defects (Table III: 1).

At 28 days postoperatively the roentgenographic examination showed substantial mineralizations throughout the defects (Figs. I: 3 and III: 3 C). The planimetric estimation indicated mineral depositions in almost the whole initial bone defect area (Table III: 1). The bone forming activity was substantially higher compared with the 14 day observations (Fig. III: 1 B; Table III: 3) and the autoradiographs showed pronounced uptake in the entire defects (Fig. III: 3 D). Histological examinations showed almost total disintegration of the original bone matrix structure and presence of newly formed immature woven bone extending throughout the defects (Fig. I: 5 A, B, C).

After three months there was still a high bone forming activity over the defects whereas the activity in the callus formation had decreased (Fig. II: 2 A). Six months after the grafting of the demineralized autogeneic bone the activity had almost ceased as registered by scintigraphy (Fig. II: 2 B) and the defects had healed with a tendency for cortical outlining and marrow space formation, both indicating structure adaptation (Fig. II: 3).

5 Experiments with composite grafts of demineralized bone and marrow (III)

Fourteen days after the operations the roentgenograms showed defects partially filled with extensive mineralizations (Fig. III: 2 A). The mineralized areas were estimated to cover about two thirds of the total defect areas (Table III: 1). Scintigraphy showed high and even bone forming activity across the defects. The activity was found to be almost as high as over the callus formations at the resection ends (Fig. III: 1 A). The bone forming activity and the amount of bone formed in the composite grafted defects was significantly higher as compared with defects grafted only with demineralized bone. Twenty-eight days after operation still higher bone (Table III: 2). Twenty-eight days after operation still higher activity was registered (Fig. III: 1 B; Table III: 3) (as compared with the 14 day observations). The roentgenograms showed extensive mineral depositions (Fig. III: 3 A) corresponding with the activities given by the autoradiograms (Fig. III: 3 B) and occupying almost the entire

defect areas (Table III: 1). Compared with the defects grafted with demineralized bone only, both the areas of bone formed and the registered activity had reached the same level.

6 Influence of growth hormone on demineralized implants (IV)

Both on the hormone- and salinetreated sides the radio-graphs showed varying degrees of mineralization at 14 days after operation (Fig. IV: 2 A and D; Table IV: 1). At both sides the scintigraphic registrations showed lower activity in the centre of the defects than at the resection ends (Fig. IV: 5; Table IV: 2). Increased activity was noticed between the 14 and 28 day observations (Figs. IV: 5 and 6; Table IV: 2) giving estimated mineralization areas of about 90 per cent both at the hormone- and salinetreated sides (Table IV: 1). No differences in the degrees or patterns of the $^{99}\text{Tc}^{\text{m}}$ uptake could be observed between the results from grafts treated with hormone and saline. In the defects remnants of the implanted bone matrix were visible (Figs. IV: 2 and 3).

7 Influence of direct currents on demineralized implants (V)

Fourteen days after operation mineralization was found in about 40 per cent of the area around the active electrodes whereas around the passive ones the corresponding value was somewhat lower (Table V: 1). The scintigraphic registration, however, showed lower bone forming activity at the sides influenced by direct currents as compared with the nonstimulated sides (Fig. V: 5; Table V: 2). In the autoradio-

grams certain negative mineralization effects were noticed around the electrodes both on the stimulated and non-stimulated (Fig. V: 4 B). The scintigraphic 28 day registrations showed increased bone forming activities (Fig. V: 6) in comparison with the 14 day results (Fig. V: 5). When the influence of the applied direct currents on bone formation was compared for the 14 and the 28 day registrations a significant increase in activity was observed (Table V: 2; Fig. V: 7) resulting in areas of mineralization of somewhat over three fourths of the total defect area (Table V: 1).

DISCUSSION

Surgical elimination of tumors in the facial region are in many cases mutilating. At the rehabilitation of such patients it is essential to try to obtain sufficient restitution of form, texture and function. As considerable problems are associated with this reconstruction the improvement of bone formation when using bone grafts must be considered clinically highly relevant. This study was carried out to explore the possibilities to optimize bone formation, especially the use of demineralized bone grafts in reconstructive treatment.

The principles found for evaluation and for stimulation of bone formation in a defect after resection are applicable to facial reconstructive surgery, but are also pertinent to bone formation in other parts of the body. Reuse of preserved bone as a substitute for autogeneic fresh bone presupposes devitalization. For this purpose autoclaving or gammara-diation are not suitable as these methods result in an almost total reduction of the inductive capacity of the bone (Buring & Urist, 1967). The demineralization process by an aqueous 0.6 N hydrochloric acid solution has, on the other hand, been shown to leave no vital cells (Urist, Silverman, Buring, Dubuc & Rosenberg 1967) and, when the grafting bone is retrieved under sterile conditions, there is no further need for treatment of the bone. Acid demineralization has also been shown to preserve the inductive properties in compact bone (Urist 1970). In this context storage at temperatures of +4⁰ C or below is also considered important to

reduce the risks for enzymatic action reducing the morphogenic response (Iwata & Urist 1972).

Graft rejections as results of immunologic reactions have resulted in attempts to reduce the antigenicity of allografts by freezing, freezedrying or chemical antigen-extraction (Chalmers 1959; Urist 1980; Urist & Dawson 1981). Although antigenicity is reduced it is by no means certain that an allograft will be successfully incorporated in the long run (Brown & Cruess 1982). An implication of the importance of grafting autogeneic demineralized bone, as suggested in paper I and II, is noticed when the results of mineralization in the grafted skin tubes 6 months postoperatively (Fig. II: 4) are compared with the results obtained after implantation of demineralized allogeneic bone in the abdominal muscle of rabbits (Urist et al. 1967). In the autogeneic heterotopically implanted bone grafts no resorption was noticed, but there was increased radiopacity compared with the three months postoperative results, indicating further mineral deposition. On the contrary the allogeneic bone grafts were gradually resorbed from the 12th week and continuously reducing in size over a period up to between 6 and 9 months.

The periosteum consists of two main cell layers with no distinct borderline. The outer layer is fibrous whilst the inner, the cambium layer, consists of competent osteoprogenitor cells (Tonna & Cronkite 1961). The periosteum is essential in bone healing and through the

osteogenetic cells it possesses a bone forming capacity of its own. In an experimental model like this it therefore has to be removed on the retrieval of the bone grafts. Otherwise the periosteum would promote healing with consequent difficulties in distinguishing between different bone-forming effects. Surrounding vital bone has a positive influence on bone formation in the bone defects (Nathansson 1977). The presence of opposing fragments in bone defects is also essential to bone healing. This implies that if interposition by fibrous tissue can be excluded, wider gaps can be bridged (Mulholland & Pritchards 1959). It is thus obvious that there is an influence both from the callus formation and adjacent vital bone. In some cases of the experimental model used in this study, overgrowth from the extensive callus into the demineralized graft was noticed on one side. On the other side, however, a space was observed between the bone endings and the grafts resembling the initial phase of pseudarthrosis formation. This reaction was believed to be due to movement of the grafts. As at the same time no decreased bone-forming activity was noticed in the graft at this side, indicating that the inductive properties of the bone matrix contributed to the healing irrespective of the callus and the adjacent bone (Figs. III: 2 B and IV: 4 D).

The roentgenographic examinations reflected the accumulated mineralization and gave information about the total amounts of bone formed inside the defects. The subjective evaluation of the roentgenograms showed to be accurate enough to

discriminate also between small differences in mineralization (III). In combination with the planimetric estimations it further showed to provide a numerical expression of differences in bone formation between the experimental and control sides (III).

The more laborious conventional histological methods used in paper I were substituted for the technetium bone-imaging as a method at the quantification of the bone forming activity in the experimental series in papers II, III, IV and V. Radionuclide imaging using $^{99}\text{Tc}^m$ or other short-lived isotopes utilizes the actual uptake as a measurement of the bone forming activity in the defect (Subramanian, McAfee, Bell, O'Mara & Ralstone 1972). The presence of or formation of hydroxyapatite is a prerequisite for the uptake of $^{99}\text{Tc}^m$ -labelled phosphorus compounds (Rohlin, Larsson & Hammarström 1977), and is thus particularly useful in studies of demineralized grafts as the uptake reveals exclusively new bone formation. Serial radionuclide images are therefore excellent diagnostic adjuncts to roentgenograms. The autoradiograms also proved to be useful supplements to both the roentgenograms and the scintigrams as they visualize in more detail the extent of uptake in different sections of the grafted area. Even if the local uptake of radionuclides is influenced by factors as blood flow, capillary permeability and bone mass (King, Klipper & Weber 1977), there is no reason to suspect the presence of systematic influence on the results in any direction in the scintigrams.

The gammacamera equipped with pin-hole collimator made it possible to focus on the target area with increased precision. With this technique it was also possible to separate the activities at the resection ends from that in the centre of the defect. This proved necessary in studies of the activities at the 14 and 28 days postoperative observations, due to the high activities at the resection ends (III, IV, V).

In the series of investigation, two different $^{99}\text{Tc}^{\text{m}}$ -labelled phosphates, MDP (methylene diphosphonate, II and III) and DPD (dicarboxypropane diphosphonate, IV and V) were used for bone scanning. DPD in comparison with MDP produces bone scans of higher quality, i.e. a better separation between soft tissue activity and bone uptake (Nosslin 1982). These two diphosphonates have however the same mechanism for uptake both in normal bone and bone lesions and there is no reason to suspect any influence on the relative values used in these studies.

Enneking (1975) analyzed the incorporation of 4 cm diameter cortical autografts in dogs and found that resorptions and porosities weakened the grafts over a period of six months. Further, the grafts were not judged to be incorporated even after one year. Nonvital bone does, however, not have to be resorbed and subsequently replaced by newly formed bone in order to function properly (Enneking 1975; Olerud & Dankwardt-Lillieström 1971). Revascularization through the Haversian systems, recanalization and invading of capillaries (Albrektsson 1979) from the host bed across

the gap between the graft and the bone ends of the defect, can gradually replace the non-vital bone and thus incorporate the graft (Olerud & Dankwardt-Lillieström 1971). Even if the donor tissue in as much as 90 per cent of the volume of cortical bone may be be unresorbed for long periods of time, for example in cases of freezetrement without excision (Emmings, Neiders, Greene, Sheldon & Gage 1966), it can still support the hard tissue structure and act as a framework. In the experimental model used in paper I, after four weeks, there were signs of extremely slow incorporation of the undemineralized bone, with only limited areas of newly formed bone (Fig. I: 7). In other rabbits the undemineralized cortical graft was unaffected and enveloped in a fibrous tissue without signs of incorporation (Fig. I: 8). In contrast, the demineralized bone grafts were almost totally resorbed and replaced by premature, woven bone four weeks after transplantation (Figs I: 5 and 6; Table I: 1). The grafts were not at this time considered as a supporting structure. From three to six months there was, however, a gradual structural adaptation (Fig. II: 3) where the cortical outlining and marrow space indicated satisfactory healing of the bone defects. It was therefore concluded that demineralization accelerates the osteogenetic process. If further indicates that the use of demineralized bone is advantageous in grafting of non-vital bone.

Though the healing capacity is far more rapid and the inherent ability to bridge large bone defects is far better in rabbits than man, the bone defects in this study were shown to have

wide enough diastasis not to heal without some kind of treatment (Fig. II: 5). Over the period of six months no healing (except in two cases) was noticed in the ungrafted defects and the scintigraphic registration revealed no substantial activity at the end of the experimental period (Fig. II: 2) indicating that no further healing was to be expected.

The presence of osteogenetic precursor cells in bone marrow has been extensively evaluated (Friedenstein 1973; Ashton, Allen, Howlett, Eaglesom, Hattori & Owen 1980). The contribution of marrow cells to the healing of composite grafts is supposed to depend more upon the complement of marrow cells than on the influence of the viable or non-viable graft according to some investigators (Burwell 1964; Nade & Burwell 1977). Lindholm and Urist (1980) have on the other hand claimed that when composite grafts of bone marrow and demineralized bone matrix are used this combination gives more bone tissue than that formed by marrow and matrix separately. The bone forming capacity of a combination of the osteogenetic demineralized compact bone and fresh autologous bone marrow could thus, when used as a composite graft, perhaps be further enhanced. This view is supported by the observation that the osteogenetic activity (III) was significantly higher at 14 days after operation in bone defects where a combination of fresh marrow cells and bone matrix had been grafted (Figs. III: 2 A and B; Tables III: 1 and 2). Obviously, in bone marrow, the osteogenetic cells can begin bone formation soon after transplantation (osteoconduction), whereas the influence

from osteoinduction (Urist 1970) is initially delayed but then gradually increased. This results in approximately the same bone forming activity and amounts of bone formed at 28 days after implantation of the demineralized bone as compared with the composite grafts of marrow and demineralized bone (Figs. III: 3 A-D; Tables III: 1 and 3). Apparently in the marrow cell population the existing osteogenetic cells influence the bone formation in the early healing process, whilst the subsequent cell generations are probably of less significance. These results also indicate that osteoinduction is an important factor in bone formation and healing of bone defects transplanted with composite grafts of fresh autologous marrow and demineralized compact autologous bone.

Growth hormone is essential for normal growth and regeneration (Thorngren 1973). The mode of this hormonal action is not known in detail but the hormone is thought to activate a series of cellular reactions leading to an increase in mitogenesis and DNA synthesis (Daughaday & Reeder 1966). In relation to bone most of the clinical research has been performed on individuals growing or with deficient amounts of growth hormone (Urist 1972). Recently Reddi and Sullivan (1980) have shown that growth hormone can regulate the proliferation and differentiation of mesenchymal bone precursor cells in normal individuals. It has also been demonstrated that growth hormone can have an accelerating effect on the osteoblastic activity (Handelsman & Gordon 1930; Frost 1962; Harris, Heaney, Jowsey, Cockin et al. 1972).

It has recently been possible to produce biosynthetical human growth hormone by gene modification techniques. Therefore in the future human growth hormone, which so far has been available only in limited amounts, may be used to enhance bone formation in bone grafting, slow healing fractures and pseudoarthroses. Though added growth hormone in available doses has shown small or no effect on the normal bone turnover in subjects with sufficient amounts of growth hormone it is still possible that bone healing could be influenced by adequate doses of growth hormone. As previously only systemic administration of growth hormone has been used, comparatively large doses therefore would be necessary. To reach sufficient local concentrations, it was thought to be of interest to study in greater detail the influence of local administration of growth hormone on bone healing, especially as direct effects of growth hormone have recently been shown to influence the longitudinal bone growth process (Isaksson, Jansson & Gause 1982). The experimental methods described in paper IV made it possible to use smaller doses and still reach sufficient local concentrations.

Urist et al. (1967) have claimed that the environment close to an implant of a demineralized lyophilized bone matrix consists of a sero-sanguineous fluid with a high concentration of proteins and cells, mainly leucocytes and wandering histocytes. This oedema persists during a period of approximately one hour to twelve days. It is then gradually resorbed and replaced by a granulation tissue with increasing amounts of mesenchymal cells which hypertrophy and gradually resemble

osteoblasts. According to the results in paper IV and V the inflammatory response, with its exudate of proteins, the local concentration of cellular elements and the biochemical environment, may be optimal for osteoinduction and subsequent bone formation. Changes in concentration of cells and eventual dilutions of biochemical components caused by the distribution of the hormone solutions through the osmotic pumps were therefore probably disadvantageous, at least at the beginning of the healing process. Furthermore, because the bone matrix protein is diffusible and hydrophobic (Urist, Mikulski & Lietze 1979) it is most likely that an increase of fluid has a negative influence of its own. Bearing this in mind it is believed that local administration of growth hormone should be deferred till twelve to fourteen days after implantation of the bone matrix. An alternative could be to use systemic administration of growth hormone in frequent doses during the induction- and bone-forming processes thereby avoiding the possible negative influence from the solution, or to concentrate the growth hormone solution thereby decreasing the flow.

Among theories on bone physiology, electrical or mechanical factors are believed to be of importance (Basset 1971). Currents have been claimed to increase osteogenesis clinically (Paterson, Lewis & Cass 1980; Basset 1982). Though both clinical and experimental studies have shown increased osteogenesis when bone is stimulated with weak electric currents the mechanism behind this enhancement of bone formation is not fully known. Brown and Cruess (1982) claim

that there is a stimulation of the collagen matrix to receive hydroxyapatite crystals whereas Basset, Chokshi, Hernandez, Pawluk and Strop (1979) suggest that there is an alteration of the proteoglycan-rich cartilage in the fibrocartilaginous tissue, bridging for example a pseudarthrosis, thereby altering the conditions for mineralization. Herbst, Lindskog and Hammarström (1981) have shown increased metabolic activity of osteogenetic cells in a tissue culture influenced by electrostimulation. Basset (1982) has recently proposed that the electromagnetic field between the anode and the cathode has varying effects on bone formation. It should thus be possible to achieve both antagonistic and synergistic effects on different levels of osteogenesis by changing for example current level, current density, polarization and wave form. Basset (1982) therefore recommended the usage of pulsing electromagnetic fields (PEMF) as these do not give rise to permanent polarization at the electrodes with consequent negative influence on the primary osteoinduction. As certain initial negative effects were observed in paper V it is possible that at least during the first 14 days they could be related to a change in the microenvironment with a deviation from the supposed optimal milieu created by the inflammatory reaction. Initially the enrichment of both organic and inorganic cations is supposed to interfere with the induction process, i.e. the degradation of the demineralized bone and the proliferation of histocytes to osteoprogenitor cells (Fig. V: 2 A; Tables V: 1 and 2). Later in the bone-forming process and related to the findings 28 days post-operatively, there is obviously an increased activity in the

cathode area. Thus, in this phase in the bone-forming process the microenvironmental changes caused by the electric currents could explain the positive effects in the cathode area (Fig. V: 2 C; Tables V: 1 and 2).

At analysis of the studied factors supposed to enhance bone formation in bone defects in rabbits, the most positive results were found in the experiment where a combination of demineralized bone matrix and fresh bone marrow was used. Local administration of growth hormone or application of direct currents to the bone matrix showed on the other hand no additional stimulating effects on bone formation. It is, however, still possible that such potential bone-growth stimulating factors could be more efficient if bone marrow cells are added. All these conditions for optimum bone formation in grafted defects will have to be considered before clinical applications of autologous demineralized bone can be made, e.g. before excised bone tissue can be reused as grafts in reconstructive treatments.

GENERAL SUMMARY

This study was undertaken to investigate factors of importance for bone formation and healing after resection, in particular the possibilities to reuse an excised piece of bone as a substitute for the commonly used autologous fresh bone transplant.

The evaluations of the progress of the mineralization process were performed through light- and fluorescence microscopy, roentgenography including planimetry and autoradiography. Of these methods scintigraphy proved to be of particular value under the experimental conditions of this study.

The results of this study show that demineralization of non-vital compact bone accelerates the bone-forming process. The subsequent formation of new bone was found to reconstitute the bone defect with a bridging hard tissue. The implanted non-vital compact bone showed few cases of incorporation. Only minor resorption and new bone formation were observed sporadically at the bone surface.

After six months the demineralized compact bone was found to have been replaced by bone tissue with cortical outlining and marrow space bridging the entire bone defect, fulfilling the criterias for healing. When the bone defects were left without grafts in most cases no healing was observed.

Autologous marrow in combination with demineralized bone was

found to accelerate the osteogenetic activity in the early stages of the healing process. Supplemented bone marrow would therefore be advantageous to use in cases where the bone implant is placed in a tissue with inferior osteoconductive properties, as are the situations for example after treatment with cytostatics and radiotherapy.

It is likely that the reaction to the operative trauma creates a favourable environment for osteoinduction and that substances used to enhance bone formation should therefore be given through systemic administration . Locally given growth hormone during the first 14 days after implantation of the demineralized bone grafts was thus not shown to have any influence on the osteogenetic activity. There was rather a delay in the resorption of the implanted matrix not observed previously. This delay was probably due to an excess of extracellular fluid causing a dilution of the active biochemical components involved in the degradation of the implanted matrix.

Electrically induced environmental changes around the implanted demineralized bone grafts were found to have a slight negative interference with the osteogenetic activity during the first 14 days after operation. During the following 14 days, however, there were indications of increased osteogenetic activity pointing to a possible use of electric currents in later stages of the bone-forming process.

ACKNOWLEDGEMENTS

I want to express my sincere gratitude to:

The late professor Sven Ingelstedt, who from his never-ceasing source of ideas initiated my interest in facial reconstructive problems;

My co-authors, Professor Per-Olof Glantz, Docent Bertil Nosslin, Docent Björn Palmer, Docent Madeleine Rohlin and Docent Karl-Göran Thorngren - in different ways my scientific tutors - for inspiring discussions, constructive criticism and skilled help and guidance;

Docent Karin Willmar-Hogeman for encouraging support and stimulating discussions throughout the years;

Mrs. Christina Lindqvist, Mrs. Barbro Wedell and Mrs. Britta Mark for valuable technical assistance;

Miss Maud Samelsson, Mrs. Birgitta Furin and Mrs. Majken Burlion for competent and patient secreterial assistance and expert typing and retyping of the manuscripts;

My daughter Anna for good photographic work and design of the figures;

My family Kerstin, Tomas, Anna and Cecilia for their support and participation throughout this study.

REFERENCES

- Albrektsson, T. Healing of bone grafts. In vivo-studies of tissue reactions at autografting of bone in the rabbit tibia. Dept. of Anatomy, Univ. of Gothenburg, Sweden. 1979.
- Ashton, B.A., Allen, T.D., Howlett, C.R., Eaglesom, C.C. Hattori, A. & Owen, M. 1980. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin. Orthop. 151, 294.
- Basset, C.A.L. 1971. Biophysical principles affecting bone structure. In: The biochemistry and physiology of bone (ed. G.H. Bourne). III, pp. 1-76. Academic Press, New York - London.
- Basset, C.A.L. 1982. Pulsing electromagnetic fields: A new method to modify cell behavior in calcified and noncalcified tissues. Calcif. Tissue Int. 34, 1.
- Basset, C.A.L., Chokshi, H.R., Hernandez, E., Pawluk, R.J.J. & Strop, M. 1979. The effect of pulsing electromagnetic fields on cellular calcium and calcification of non-unions. In: Electrical properties of bone and cartilage. Experimental effects and clinical application (eds. C.T. Brighton, J. Black & S.R. Pollack). pp 427-441. Grune & Stratton, New York.
- Brown, K.L.B. & Cruess, R.L. 1982. Bone and cartilage transplantation in orthopaedic surgery. J. Bone Joint Surg. 64-A, 270.
- Burchardt, H., Glowziewskie, F.P. & Enneking, W.F. 1977. Allogenic segmental fibular transplants in azathioprine immunosuppressed dogs. J. Bone Joint Surg. 59-A, 7.

- Buring, K. & Urist, M.R. 1967. Effects of ionizing radiation on the bone induction principle in the matrix of bone implants. Clin. Orthop. 55, 225.
- Burwell, R.G. 1964. Studies in the transplantation of bone; VII. The fresh composite homograft-autograft of cancellous bone; an analysis of factors leading to osteogenesis in marrow transplants and marrow-containing grafts. J. Bone Joint Surg. 46-B, 110.
- Chalmers, J. 1959. Transplantation immunity in bone homografting. J. Bone Joint Surg. 41-B, 160.
- Daughaday, W.H. & Reeder, C. 1966. Synchronous activation of DNA synthesis in hypophysectomized rat cartilage by growth hormone. J. Lab. Clin. Med. 68, 357.
- Emmings, F.G., Neiders, M.E., Greene, G.W., Jr., Sheldon, W.K. & Gage, A.A. 1966. Freezing the mandible without excision. J. Oral Surg. 24, 145.
- Enneking, W.F. 1975. Physical and biological aspects of repair in dog cortical bone transplants. J. Bone Joint Surg. 57-A, 237.
- Friedenstein, A.J. 1973. Determined and inducible osteogenic precursor cells. In: Hard Tissue Growth Repair and Remineralization. Ciba Foundation Symposium II. New York, Elsevier, pp 169-181.
- Friedlaender, G.E. 1982. Current concepts review bone-banking. J. Bone Joint Surg. 64-A, 307.
- Frost, H.M. 1962. A model of endocrine control of bone remodelling. Henry Ford Hosp. M. Bull. 10, pt. 2, 119

- Gage, A.A., Greene, G.W., Jr., Neiders, M.E. & Emmings, F.G. 1966. Freezing bone without excision: an experimental study of bone-cell destruction and regrowth. J.A.M.A. 196, 90.
- Handelsman, M.B. & Gordon, E.F. 1930. Growth and bone changes in rats injected with anterior pituitary extract. J. Pharmacol. Exp. Ther. 38, 349.
- Harris, W.H., Heaney, R.P., Jowsey, J., Cockin, J., Akins, C., Graham, J. & Weinberg, E.H. 1972. Growth hormone: The effect on skeletal renewal in adult dog. Calcif. Tissue Res. 10, 1.
- Herbst, E., Lindskog, S. & Hammarström, L. 1981. A method for electrical stimulation in vitro. Technical Report 3:81. Res. Lab. Med. Electr. Chalmers University of Technology, Göteborg, Sweden.
- Holtfreter, J. 1933. Nachweis der Induktionsfähigkeit abgetöteter Keimteile. Isolations und Transplantationsversuche. Arch. Entwickl.-Mech. Org. 128, 584.
- Isaksson, O.G.P., Jansson, J.-O. & Gause, I.A.M. 1982. Growth hormone stimulates longitudinal bone growth directly. Science 216, 1237.
- Iwata, H. & Urist, M.R. 1972. Protein polysaccharide of bone morphogenetic matrix. Clin. Orthop. 87, 257.
- King, M.A., Klipper, R.W. & Weber, D.A. 1977. A model for local accumulation of bone imaging radiopharmaceuticals. J. Nucl. Med. 18, 1106.
- Lindholm, T.S. & Urist, M.R. 1980. A quantitative analysis of new bone formation by induction in composite grafts of bone marrow and bone matrix. Clin. Orthop. 150, 288.

- Marciani, R.D., Giansianti, J.S. & Massey, G.B. 1967. Reimplantation of freezetreated and saline-treated mandibular bone. *J. Oral Surg.* 34, 314.
- Marcove, R.C. & Miller, T.R. 1969. Treatment of primary and metastatic bone tumors by cryosurgery. *J.A.M.A.* 207, 1890.
- Merz, W.A. & Schenk, R.K. 1970, Quantitative structural analysis of human cancellous bone. *Acta Anat.* 75, 54.
- Mizutani, H. & Urist, M.R. 1982. The nature of bone morphogenetic protein (BMP) fractions derived from bovine bone matrix gelatin. *Clin. Orthop.* 171, 213.
- Mulholland, M.C. & Pritchard, J.J. 1959. The fracture gap. *J. Anat.* 93, 590.
- Muscolo, D.L. & Kawai, S. 1977. Bone transplantation antigens. Cellular and humoral immune response studies. *International orthopaedics (SICOT)* 1, 5. Springer-Verlag.
- Muscolo, D.L., Kawai, S. & Roy, R.D. 1977. In vitro studies of transplantation antigens in the rat. *J. Bone Joint Surg.* 59-B, 510.
- Nade, S. & Burwell, R.G. 1977. Decalcified bone as a substrate for osteogenesis. *J. Bone Joint Surg.* 59-B, 188.
- Nathansson, A. 1977. The early vascularization of an autologous bone inlay into an artificial defect in the rabbit mandibula. *Acta Otolaryngol.* 84, 1.
- Nosslin, B. 1982. A clinical comparison of DPD and MDP for bone scanning. In: *Nuklearmedizin. Computergestützte funktionelle Analyse.* (eds. H.A.E. Schmidt & H. Rösler). F.K. Schautauer, Stuttgart-New York, p.923

- Olerud, S. & Dankwardt-Lillieström, G. 1971. Fracture healing in compression osteosynthesis. Acta Orthop. Scand. Suppl. 137.
- Osborne, D.B., Lilly, G.E., Thompson, C.W. & Jost, T. 1977. Bone grafts with surface decalcified allogenic and particular autologous bone: report of cases. J. Oral Surg. 35, 276.
- Parrish, F.F. 1973. Allograft replacement of all or part of the end of a long bone following excision of a tumor. Report of twenty-one cases. J. Bone Joint Surg. 55-A, 1
- Paterson, D.C., Lewis, G.N. & Cass, C.A. 1980. Treatment of delayed union and nonunion with an implanted direct current stimulator. Clin. Orthop. 148, 117.
- van de Putte, K.A. & Urist, M.R. 1965. Osteogenesis in the interior of intramuscular implants of decalcified bone matrix. Clin. Orthop. 43, 257.
- Ramani, P.S., Kalbag, R.M. & Sengupeta, R.P. 1975. Cervical spinal interbody fusion with Kiel bone. Br. J. Surg. 62, 142.
- Reddi, A.H. & Sullivan, N.S. 1980. Matrix induced endochondral bone differentiation: Influence of hypophysectomy, growth hormone, and thyroid stimulating hormone. Endocrinology 107, 1291.
- Rohlin, M., Larsson, Å, & Hammarström, L. 1977. Distribution of $^{99}\text{Tc}^{\text{m}}$ -labelled phosphorus compounds, ^{45}Ca and ^{85}Sr in diphosphonate-treated rats. Acta radiol. Phys. Biol. 16, 513.

- Senn, N. 1889. On the healing of aseptic bone cavities by implantation of aseptic decalcified bone.
Am. J. Med. Sciences 98, 219. Reviewed in Chase, S.W. & Herndon, Ch. H. The fate of autogenous and homogenous bone grafts. A historical review. J. Bone Joint Surg. 37-A, 809.
- Smith, D.B. & Weaver, A.W. 1976. Cryosurgery for oral cancer, a six year retrospective study. J. Oral Surg. 34, 245.
- Subramanian, G., McAfee, J.G., Bell, E.G., Blair, R.J., O'Mara, R.E. & Ralston, P.H. 1972. ^{99m}Tc -labeled polyphosphate as a skeletal imaging agent. Radiology 102, 701.
- Thorngren, K.-G. 1973. Growth hormone and longitudinal bone growth. Comm. Dept. Anat. (Lund) 10, pp. 1-39.
- Tonna, E.A. & Cronkite, E.P. 1961. Cellular response to fracture studied with tritiated thymidine. J. Urist, M.R. 1970. The substratum for bone morphogenesis. Dev. Biol. Suppl. 4, 125.
- Urist, M.R. 1972. Growth hormone and skeletal tissue metabolism. In: Biochem. and Physiol. of Bone Vol II (ed. G.H. Bourne) Academic Press, New York, London, pp. 155-195.
- Urist, M.R. 1980. Bone transplants and implants. In: Fundamental and Clinical Bone Physiology, Chapter 11, pp. 331-368, Philadelphia, J.B. Lippincott.
- Urist, M.R. & Dawson, E. 1981. Intertransverse process fusion with the aid of chemosterilized autolyzed

antigen-extracted allogeneic bones. Clin. Orthop.

154, 97.

Urist, M.R., Mikulski, A.J. & Lietze, A. 1979.

Solubilized and insolubilized bone morphonetic
protein. Proc. Natl. Acad. Sci. 76, 1828.

Urist, M.R., Silverman, B.F., Buring, K., Dubac, F.L.

& Rosenberg, J.M. 1967. The bone induction principle.
Clin. Orthop. 53, 243.

Williams, G. 1964. Experience with boiled cadaveric

cancellous bone for fracture of long bones. J. Bone
Joint Surg. 46, 398.

OSTEOGENETIC PROPERTIES OF REIMPLANTED DECALCIFIED AND UNDECALCIFIED AUTOLOGOUS BONE IN THE RABBIT RADIUS¹

J. Wittbjer, B. Palmer and K.-G. Thorngren

From the Maxillofacial Center (Head: K.-V. Sarnäs) and Department of Plastic Surgery (Head: S. Jacobsson), Malmö General Hospital, the Department of Prosthetic Dentistry (Head: P.-O. Glantz), and the Department of Orthopaedic Surgery (Head: G. C. H. Bauer), University of Lund, Sweden

(Submitted for publication March 16, 1982)

Abstract. In facial reconstructive surgery extensive studies have been performed to find substitutes to autologous vital bone as grafting material. Decalcified homologous compact bone has been found to possess a capacity of inducing new bone formation in rabbits whereas the application in man has not been successful thus far. In order to investigate the osteogenetic properties in autologous devitalized bone, the difference in bone forming capacity between decalcified and undecalcified devitalized autologous bone was studied. In rabbits, from the radius of each foreleg a piece of bone was resected, rinsed from periosteum and marrow whereupon one was decalcified in 0.6 N HCl. Both pieces were reimplanted in the radial defects 3 days later. The healing process was examined by radiography, light- and fluorescence microscopy. Decalcification seems to accelerate the osteogenetic process when bone is reimplanted to a defect in the rabbit radius.

Barth (1898) and later Axhausen (1907) stated that in transplantation of viable bone including periosteum, the periosteum will survive, retain its capacity of ossification while the bone and marrow can be considered as non vital. This theory concerning compact bone grafts was later confirmed by Ham (1952). During the last 20 years preserved homologous bone, deepfrozen or lyophilized, compact or cancellous has been used as substitute to autologous bone (Chase & Herndon, 1955; Burwell, 1966; Glowacki, Altobelli & Mulliken, 1981).

In facial reconstructive surgery e.g. after tumor resection including part of the jaw, considerable bone grafting problems arise. Reconstruction of form and function are essential. Reuse of the patient's own resected bone i.e. the mandible, would be the best material for reconstruction, perfectly fitting, lacking antigenous properties and with no

additional surgical procedure needed in order to retrieve a grafting material.

•Undecalcified, devitalized bone is generally considered not to produce new bone when implanted into a muscle, whereas extensive studies have shown decalcified cortical bone to possess this capacity (Urist, 1970). Thus, decalcification of cortical bone results in collagen matrix with an inherent bone inductive capacity.

The aim of this investigation has been to determine whether there is a difference in osteogenetic properties between decalcified and undecalcified autologous bone when reimplanted in an operatively created resection defect in the rabbit radius.

MATERIAL AND METHODS

In 13 adult male rabbits with an average bodyweight of 4.1 kg, a 12 mm piece of the radius was bilaterally resected. The wounds were thoroughly rinsed by irrigation with saline in order to remove remaining bone chips. Sterile silastic tubes (Nelaton® catheter) with the dimensions corresponding to the resected bone pieces were placed in the defects and the wounds were closed.

The resected pieces of bone were rinsed from periosteum and bone marrow whereupon one was decalcified in 0.6 N HCl for 24 hours and then repeatedly rinsed in saline. After rinsing and storage for another 2 days in saline at +4°C pH was measured to 6.8. The other piece of bone was treated in the same way with the exception of decalcifying.

A second operation was performed 3 days later, the

¹ Supported by the Faculties of Odontology and Medicine University of Lund, by Greta and Johan Kocks Stiftelse, and by the Swedish Medical Research Council (Project No. 17X-2031).



Fig. 1. Roentgenograms of the forelegs 1 week after reoperation.

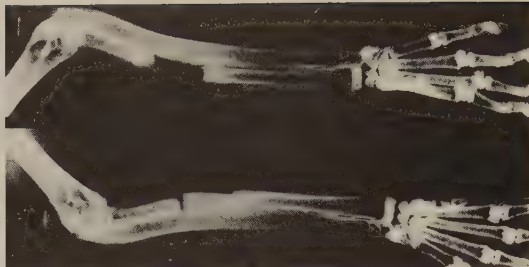


Fig. 2. Roentgenograms 3 weeks after reoperation.

plastic tubes removed and the two pieces of radius replaced in their defects. Roentgenographic examination of the healing process was performed at 7, 14 and 21 days postoperatively. The animals were sacrificed 4 weeks postoperatively. Two days before, Terramycin® vet. 1 ml/kg bodyweight at a concentration of 1 mg/ml was administered intravenously.

The forelegs were amputated 10 mm distal and proximal to the defect. Soft tissues were removed and roentgenograms of the specimens were made. Guided by these roentgenograms the specimens were sectioned into two pieces of equal size. One half was put in formaline (10%) for light microscopy examination. This part was decalcified, serially sectioned and stained with haematoxylin and eosin. The other half was put in absolute alcohol, embedded in plastic, cut transversely by a grinding plate (Stenström, Hansson & Thorngren, 1977) and examined by fluorescence microscopy. A grid according to Merz & Schenk (1970) was used for morphometric evaluation of active bone formation. The volumetric density of OTC deposition (indicating new bone formation) was determined in percentage.

RESULTS

Two rabbits suffered leg fracture and a third died during anaesthesia for roentgenographic examination.

tion, and were thus excluded, leaving 10 animals for evaluation.

Roentgenographic examination. Roentgenograms made after one week were used mainly to confirm the position and the decalcification of the grafts. There were no signs of mineralization at this time either in the decalcified graft or in the defects (Fig. 1). Roentgenograms made two weeks postoperatively indicated a reactive callus formation at the sites of amputation. In addition, the implanted decalcified bone matrix showed thin radiopaque bands in some cases. Defects with nondecalcified implanted bone had traces of periosteal callus formation at the sites of amputation but no changes within the implant.

Roentgenograms of the defects with decalcified implants three weeks postoperatively showed further mineralization (Fig. 2), however, with relatively large differences in the degree of radiopacity among the animals. In all but one case, defects with undecalcified implants showed less mineralization compared to the decalcified implants. In one case only, the undecalcified implant seemed to be included in a callus formation bridging the defect. Within

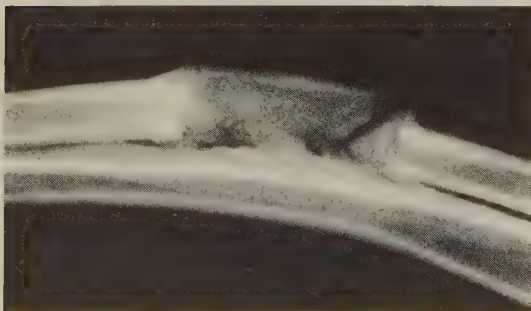


Fig. 3. Roentgenogram of the matrix specimen 4 weeks postoperatively.

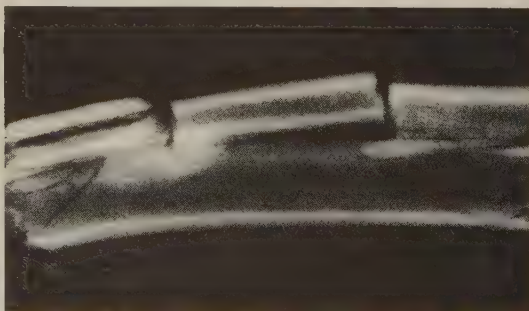


Fig. 4. Roentgenogram of undecalcified implanted bone 4 weeks postoperatively.

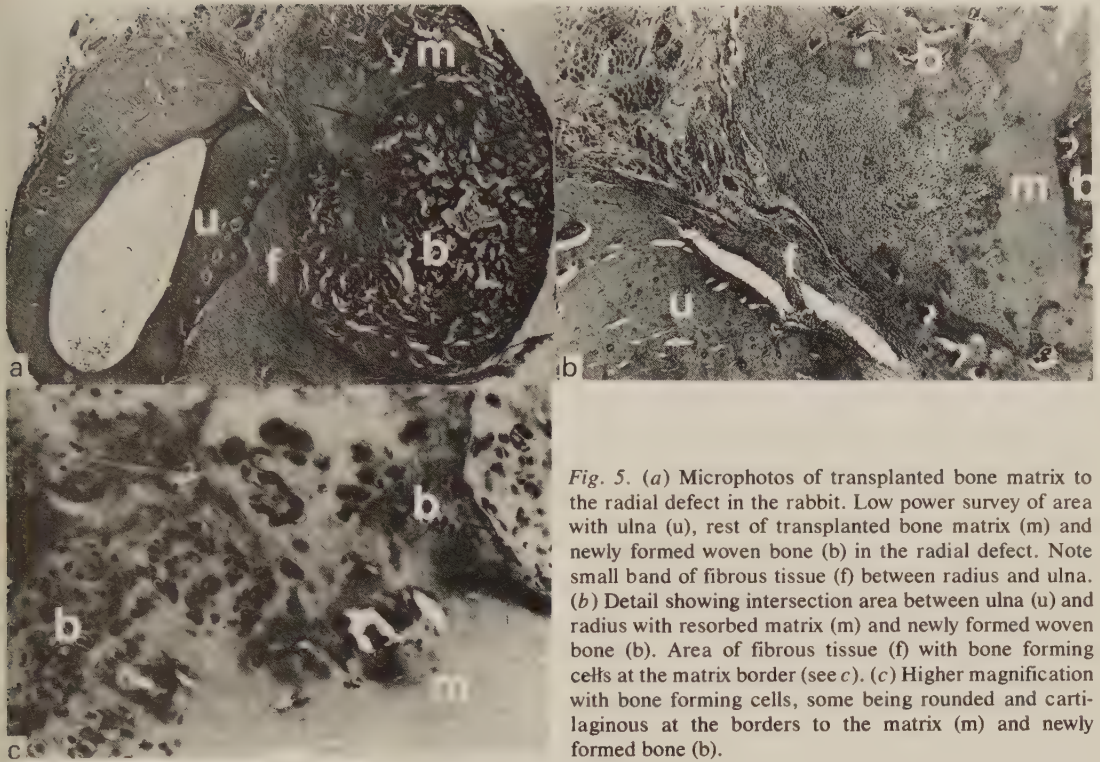


Fig. 5. (a) Microphotos of transplanted bone matrix to the radial defect in the rabbit. Low power survey of area with ulna (u), rest of transplanted bone matrix (m) and newly formed woven bone (b) in the radial defect. Note small band of fibrous tissue (f) between radius and ulna. (b) Detail showing intersection area between ulna (u) and radius with resorbed matrix (m) and newly formed woven bone (b). Area of fibrous tissue (f) with bone forming cells at the matrix border (see c). (c) Higher magnification with bone forming cells, some being rounded and cartilaginous at the borders to the matrix (m) and newly formed bone (b).

that undecalcified implant, however, no changes of mineralization or resorption were observed.

Roentgenographic examination of the specimens. Roentgenographic examination of the specimens was used for evaluation. The roentgenograms were of a higher quality and had better resolution due to a closer focus-object distance and the removal of soft tissue from the specimens.

The defects implanted with decalcified bone had a substantial mineralization corresponding to the matrix part, in some cases tending to bridge the defect (Fig. 3). However, the radiopaque structures in the matrix showed less density than in the adjacent bone tissue.

The undecalcified implants were unchanged and showed no sign of either resorption or new bone formation within the implants. No fusion was observed between these implants and the surrounding bone tissue (Fig. 4). In one case the undecalcified bone implant was included in a callus formation bridging the defect.

Histologic examination. The decalcified bone implants showed an almost total disintegration of the original matrix structures. An immature woven

bone with many osteocytes, and in some places cartilage cells, extended throughout the implant. The lack of original osteon formation indicated a rebuilding of the bone matrix. Traces of non vital bone matrix not completely resorbed, with empty or pycnotic cell nuclei were seen (Fig. 5). Some specimens indicated further disintegration and bone forming with only insignificant remnants of bone matrix in a structure otherwise resembling an ossicle (Fig. 6). One specimen showed a solid infiltration of inflammatory cells with a collagen matrix almost totally resorbed and without any traces of osteogenesis.

The undecalcified bone implants showed small islands of newly formed bone only within limited areas in the periphery (Fig. 7). In some of the specimens a fibrous tissue encapsulated the bone implant without traces of resorption or new bone formation (Fig. 8). In order to receive quantitative information concerning the amount of newly formed bone, an estimation of the area of resorption and bone formation in the implants was performed. The decalcified bone implants resulted in a significantly higher bone formation ($p < 0.05$) and



Fig. 6. Microphotographs of transplanted bone matrix to radial defect in the rabbit. (a) Massive bone formation (b) with only small remnants of unresorbed bone matrix in the radius. Periosteal bone remodelling in the ulna (u). (b) Higher magnification showing unresorbed bone matrix (m) and trabeculae of newly formed bone (b).

resorption ($p < 0.01$) over the major part of the bone implants (Table I).

Fluorochrome. Ground sections of the implanted decalcified bone gave an irregularly orientated fluorescence under fluorescence microscopy indicating bone formation in a three-dimensional network.

In all cases except one, the fluorescence areas were of less extension in the undecalcified specimens than in the decalcified ones. In one case only the bone formation was almost equal on both sides. On the sides with the decalcified bone matrix implants, the bony trabeculae with active bone formation as indicated by apposition of oxytetracycline (OTC), averaged 47 % of the visible area. On the sides with undecalcified bone implants, the OTC deposition averaged 35 %.

DISCUSSION

The organic matrix in bone-tissue is considered to have a bone inductive capacity (Urist). Whether new bone is formed by cells with bone forming capacity already existing in the tissue or by influence on undifferentiated mesenchymal cells has been the subject of discussion for a long time. The most favoured opinion is that an undifferentiated histiocyte has the inherent possibility to transform

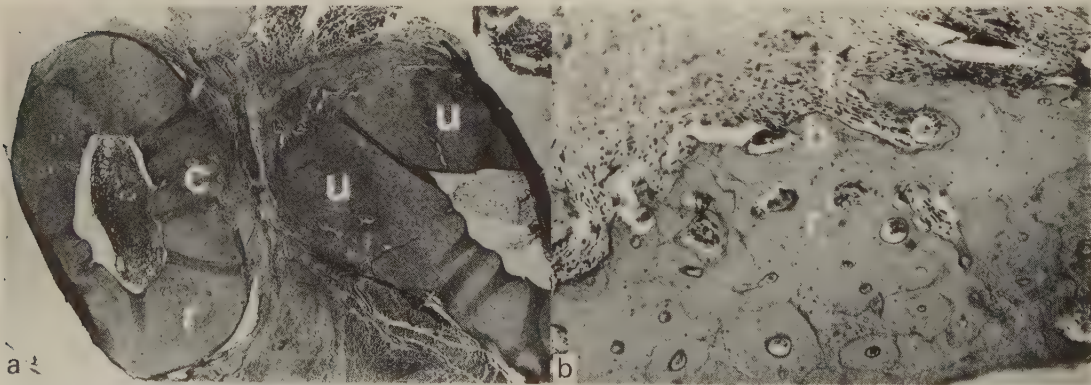


Fig. 7. Microphotographs of transplanted undecalcified bone to the radial defect in the rabbit. (a) Low power survey of area with ulna (u) and unaffected bone transplant of radius (r) with minor resorption cavities (c), (b) Detail showing intersection area between transplanted radius (r) and fibrous tissue (f) with cavities (c) of bone resorption with small bands of bone formation (b).

Table I. Bone formation and resorption in decalcified and undecalcified implants, evaluated in haematoxylin-eosin sections

Area expressed as part of total cross section area of the implant. Numbers given in the table are the animals in each category

Area	Bone formation			Implant resorption		
	Decalcif.	Signif.	Undecalcif.	Decalcif.	Signif.	Undecalcif.
0	0	***	8	0	***	8
≤1/3	4	NS	1	2	NS	1
≤2/3	0	NS	0	0	NS	0
>2/3	5	*	0	7	**	0

Statistical analysis by χ^2 test with Yate's correction:

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, NS $p > 0.05$

into a bone forming cell, an osteoblast (Urist & McLean, 1952; Urist, Wallace & Adams, 1965). This bone induction principle (BIP) is defined as an interaction between extra-cellular products from mesenchymal cells and chemical components in the substrate. When breaking down the organic matrix of the bone tissue, an unknown biochemical substance—bone morphogenetic protein (BMP) influences an undifferentiated histiocyte to mobilize its bone forming capacity (Urist, Silverman, Buring, Dubac & Rosenberg, 1967). Living cells in the matrix are consequently not a prerequisite for this process.

The method of preparation used in these investigations—decalcification in 0.6 N HCl—leaves no surviving cells in the collagen matrix (Urist et al., 1967).

The surrounding bone influences the bone forming process positively (Nathansson, 1977), and whether the healing of the bone defect in this investigation occurs without any matrix implanted is the subject of a further experiment.

Healing of a bone transplant implies a resolution of the mineral component, total rebuilding of the organic structure and a remineralization (Ham & Harris, 1971). In large transplants the central parts often disintegrate, resulting in lack of bone forming capacity (Mohnac, 1969). Consequently, small grafts have a better prognosis by a faster revascularization. Decalcification facilitates the penetration of the cell-rich serous exudate caused by surgical trauma, as well as the growth of vessels into the implant. Both these phenomena are a prerequisite of bone induction (Urist; Trueta, 1974).

The present investigation showed that decalcified bone stimulates new bone formation when implanted into a created bone defect in rabbits. It also

demonstrated that decalcification of bone prior to implantation in a defect accelerated the osteogenesis compared to undecalcified bone (Table I).

Specialization of a mesenchymal stem cell can be determined by external nutrient factors (Hall & Shorey, 1968). Adequate oxygen tension and other nutritional and physical factors (Basset, 1971) are of importance in the bone forming process. Changes in the environment can also alter the osteogenesis to chondrogenesis in the sense that diminished nutrition gives a predomination of chondrogenesis (Ham, 1930; Basset, 1964). The possible variation in oedema following surgical trauma or postoperative infection might cause a disturbance in nutrition during the early sensitive part of the osteogenetic process and can be an explanation to the various amount of chondrocytes in some sections and the difference in amount of bone formed between different animals (Fig. 5c.).

In some cases there was a synostosis between

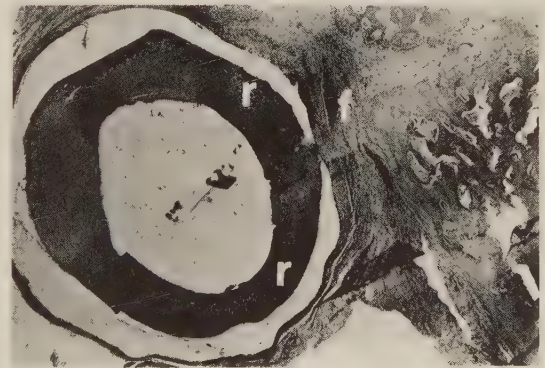


Fig. 8. Microphoto of transplanted undecalcified bone to the radial defect in the rabbit showing lack of integration between radius (r) and surrounding fibrous tissue (f) resulting in loosening artefact during sectioning.

radius and ulna, resulting in periosteal rupture at removal of the bone tissue, probably causing a reactive bone forming process accelerating the healing process. It might be the explanation as to why, in one case, remineralization of the defect occurred leaving the undecalcified bone implant adjacent to the newly formed bone without any sign of change in the structure. Despite variations in the osteogenetic process it was noticed that in defects with decalcified bone implants there was, in all cases, bone formation quantitatively and qualitatively superior to the undecalcified implant (Table I).

The evaluated degree of mineralization corresponded when determined in roentgenograms of the specimens, ground sections in fluorescence microscopy and hematoxylin-eosin stained sections in light microscopy. A more detailed quantitative determination, however, is rendered more difficult by the complex three-dimensional pattern of the bone formation.

In conclusion, when bone is retransplanted to a defect in the rabbit radius decalcification seems to accelerate the osteogenetic process, compared to undecalcified bone implants. Infection delays or disturbs the osteogenetic process. There is a good correspondence between roentgenographic examination, fluorescence and light microscopy when used for qualitative evaluation of the bone forming capacity.

REFERENCES

- Axhausen, G. 1907. Histologische Untersuchungen über Knochen-Transplantation am Menschen. *Dtsch Z Chir* 91, 388.
- Barth, A. 1898. Die Entstehung und das Wachsthum der freien Gelenkkörper. Eine histologisch-klinische Studie. *Arch Klin Chir* 56, 507.
- Basset, C. A. L. 1964. Environmental and cellular factors regulating osteogenesis. In *Biodynamics* (ed. H. M. Frost.), p. 233. Little, Brown and Co., Boston.
- 1971. Biophysical principles affecting bone structure. In *The biochemistry and physiology of bone* (ed. G. H. Bourne), vol. 3, 2nd edn., p. 1. Academic Press, New York.
- Burwell, R. G. 1966. Studies in the transplantation of bone. VIII. Treated composite homografts-autografts of cancellous bone: an analysis of inductive mechanisms in bone transplantation. *J Bone Joint Surg* 48-B, 532.
- Chase, S. & Herndon, C. H. 1955. The fate of autogenous and homogenous bone grafts. *J Bone Joint Surg* 37-A.
- Glowacki, J., Altobelli, D. & Mulliken, J. B. 1981. Fate of mineralized and demineralized osseous implants in cranial defects. *Calcif Tissue Int* 33, 71.
- Hall, B. K. & Shorey, C. D. 1968. Ultrastructural aspects of cartilage and membrane bone differentiation from common germinal cells. *Aust J Zool* 16, 821.
- Ham, A. W. 1930. A Histological study of the early phase of bone repair. *J Bone Joint Surg* 12, 825.
- 1952. Some histophysiological problems peculiar to calcified tissues. *J Bone Joint Surg* 34 A, 701.
- Ham, A. W. & Harris, W. R. 1971. Repair and transplantation of bone. In *The biochemistry and physiology of bone* (ed. G. H. Bourne), vol. 2, 2nd edn., pp. 379. Academic Press, New York.
- Merz, W. A. & Schenk, R. K. 1970. Quantitative structural analysis of human cancellous bone. *Acta Anat* 75, 54.
- Mohnac, A. M. 1969. Gross loss of mandibular hard structure. *J Oral Surg* 27, 508.
- Nathansson, A. 1977. The early vascularization of an autologous bone inlay into an artificial defect in the rabbit mandibula. *Acta Otolaryngol* 84, 1.
- Stenström, A., Hansson, L. I. & Thorngren, K. G. 1977. Cortical bone remodelling in normal rat. *Calcif Tiss Res* 23, 161.
- Trueta, J. 1974. The role of the vessels in osteogenesis. *J Bone Joint Surg* 45-B, 402.
- Urist, M. R. 1970. Substratum for bone morphogenesis. *Develop Biol Suppl* 4, 125.
- Urist, M. R. & McLean, F. C. 1952. Osteogenetic potency and new bone formation by induction in transplants to the anterior chamber of eye. *J Bone Joint Surg* 34-A, 443.
- Urist, M. R., Silverman, B. F., Buring, K., Dubac, F. L. & Rosenberg, J. M. 1967. The bone induction principle. *Clin Orthop* 53, 243.
- Urist, M. R., Wallace, T. H. & Adams, T. 1965. The function of fibrocartilaginous callus. *J Bone Joint Surg* 47-B, 304.

BONE FORMATION OF TRANSPLANTED AUTOLOGOUS BONE MATRIX IN RABBIT EVALUATED BY TECHNETIUM RADIONUCLIDE BONE IMAGING¹

J. Wittbjer, B. Nosslin, B. Palmer and K.-G. Thorngren

*From the Maxillofacial Center and Departments of Nuclear Medicine and Plastic Surgery,
Malmö General Hospital and Departments of Orthopaedic Surgery
and Prosthetic Dentistry, University of Lund, Sweden*

(Submitted for publication February 16, 1981)

Abstract. In adult rabbits a 12 mm piece of the radius was bilaterally resected and subsequently decalcified. One piece was placed in one of the radial defects and the other in a skin tube on the back. The other radial defect served as a control. The bone formation process was evaluated by roentgenographic examination and quantified radionuclide bone imaging by technetium diphosphonate scintigraphy at 3 and 6 months postoperatively. Reliability of the scintigraphy method was assessed by means of testing interexamination variability where the same sample was measured twice and where no significant difference was found. In the defect restituted with decalcified bone matrix there was a significantly ($p < 0.01$) higher bone formation rate at 3 months postoperatively, with a mean increase of 47% compared to the control side. In the skin tube the implanted matrix showed mineral formation both at 3 and 6 months postoperatively and increased radionuclide uptake compared to the adjacent tissues. Technetium radionuclide imaging was found to be a suitable method for evaluation of the bone formation in these small defects.

Treatment of tumors adjacent to bone or within bone i.e. the mandible often results in excision. In most cases partial resection is considered to give sufficient margin to tumor-involved tissue which simplifies the reconstruction (Marchetta, Sako & Murphy, 1971). In advanced cases, however, so much bone has to be excised that the mandibular continuity is interrupted resulting in facial disfigurement and malocclusion. For rehabilitation sufficient restitution of function and esthetic configuration is important. Consequently extensive experimental and clinical studies have been performed. Methods described are frameworks of metal of different design (Schmoker, Spiessl & Mathys, 1976; Austerman, Becker, Bühning & Machtens, 1977)

silastic implants (Scheunemann, 1976) resection and replantation after boiling of the mandibular bone (Williams, 1964) or freezing (Emmings, Neiders, Greene, Sheldon & Gage, 1966; Gage, Greene, Neiders & Emmings, 1966; Marcove & Miller, 1969; Smith & Weaver, 1976). Recently Lindström, Brånemark and Albrektsson (1981) have published a method for reconstruction of a mandibular defect by using osseointegrated titanium implants (Brånemark, Breine, Adell, Hansson, Lindström & Ohlsson, 1969; Albrektsson, Brånemark, Hansson & Lindström, 1981) inserted in tibial or iliac grafts allowing subsequent restoration with bridge constructions. Other types of bone grafting materials used for the purpose of reconstruction are homografts prepared in different ways to reduce the level of antigenicity (Parrish, 1973; Marciani, Giansanti & Massey, 1975; Osborn, Lilly, Thompson & Jost, 1977). The opinion concerning the antigenicity differs, however. In experimental models bone homografts have been shown with different immunological techniques to have very weak antigen-antibody reactions according to Kreuz, Hyatt, Turner & Bassett, 1951; Chalmers, 1959; Burwell, Gowland & Dexter, 1963; Elves, 1974. Despite preparation of the homografts with deepfreezing, lyophilization or decalcification weak antigenous properties remain in the collagenous matrix as found by Burchardt, Glowzewske & Enneking,

¹ Supported by the Faculties of Odontology and Medicine, University of Lund, by Trygg-Hansas Forskningsfonder and by the Swedish Medical Research Council (Project No. 17X–2031).

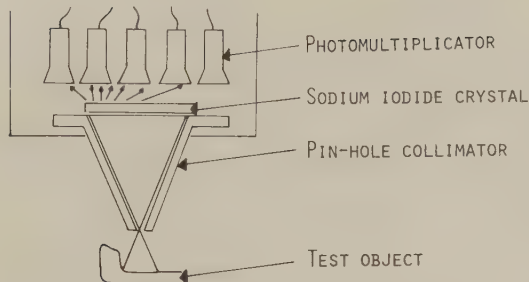


Fig. 1. Gamma-camera.

1977; Musculo & Kawai, 1977; Musculo, Kawai & Roy, 1977. Consequently attempts to induce bone with decalcified homologous bone matrix (Urist, 1970) in dog and man have not been successful (Buring, 1974). To avoid this rejection mechanism as a cause for failure autologous bone is preferable.

The aim of this investigation was to evaluate the possibility to reuse resected demineralized bone tissue as a delayed graft and a framework for bone formation.

MATERIAL AND METHODS

In eleven adult male rabbits with an average body weight of 3.8 kilograms a 12 mm piece of the radius was bilaterally resected in Mebumal anaesthesia (Nembutal vet.[®]) supplemented with local anaesthesia (Xylocain[®] 0.5 %).

The bone including periosteum was sawn off during continuous irrigation with saline. A piece of sterile plastic tube (Nelaton[®] catheter) with the dimensions corresponding to the resected bone was placed in the radial defect and the wound was closed. The two pieces of bone were rinsed from periosteum and bone marrow, whereupon they were decalcified in 0.6 N HCl for 24 hours and then repeatedly rinsed in saline. They were kept in 50 ml saline (pH 6.5) at +4°C for two days. At a second operation the wounds were bilaterally opened and the tubes taken away. One piece of decalcified bone matrix was put back into the radial defect of the left leg. The defect in the right foreleg was left empty to serve as a control.

On the back of the rabbit two parallel cuts 70 mm in length and spaced 50 mm were placed. Dissection was performed down to the fascia of the dorsal muscles layer and a skin flap was mobilized. The other decalcified piece of bone was placed in a pouch created by the musculus panniculus carnosus in the flap which was sutured into a skin tube.

The remineralisation process was evaluated by roentgenographic examination and radionuclide bone imaging by technetium diphosphonate at three and six months postoperatively. The radiographs of the bone specimens were taken with Philips Oralix apparatus (65 kV, 10 mA) on Kodak DF 57 film.

For scintigraphy ⁹⁹Tc^mMDP (Methylene-diphosphonate) was used. ⁹⁹Tc^m-pertechnetate (AB Atomenergi, Studsvik, Sweden) was added to a MDP-kit (Kabi Diagnostica, Sweden) containing 5.0 mg MDP and 0.84 mg SnCl₂. After addition of pertechnetate the solution was diluted up to 6 ml. From this about 2 ml containing an activity of 180 MBq (≈5 mCi) was given intravenously to the rabbits via an ear vein three hours before the investigation.

For examination the anaesthetized rabbit was placed in supine position on a mobile table with the forelegs fixed horizontally. A gamma-camera with pinhole collimator (6 mm diameter, length 35 cm) was focused on the area of interest at a standardized distance (65 mm from the objects upper surface to the collimator opening). This distance allowed a picture of the radius, the ulna, the elbow and most of the humerus of the rabbit (Fig. 1). The emitted gamma-radiation from the object was registered in the camera connected to a computer. With the aid of the projected picture in an oscilloscope the forelegs were directed to fit into a right angle coordinate system to facilitate quantification of the activity. A matrix containing 128×128 picture elements (pixels) was used and 100 000 counts were collected during an exposure time of about 10 min. The numerical data were stored in a disc memory.

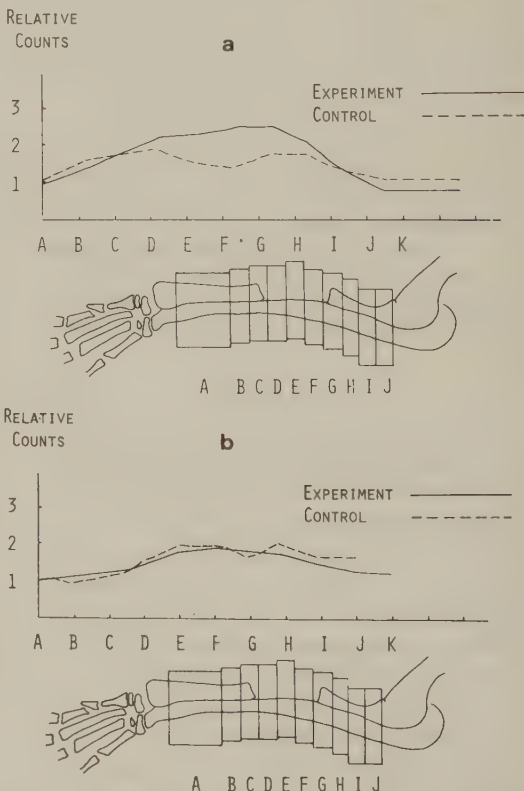


Fig. 2. γ -Radiation activity measured within regions of interest (a) 3 months postoperatively and (b) 6 months postoperatively.

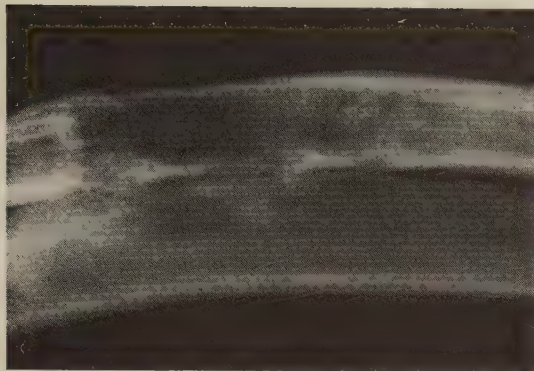


Fig. 3. The grafted radial defect 6 months postoperatively.

Regions of interest were mapped out on the computer-visualized picture (Fig. 2). A control area including 300 pixels was placed at a distance of 10 mm distal to the defect, to avoid influence by callus formation. From the control area about 10 further areas, each including 100 pixels were placed consecutively in the proximal direction. Mean counts in every region of interest were measured separately and the differences to the control area were calculated. Relative values to the control area were then calculated for each region and added.

Determination of the error of method was made by randomized double determinations at the same investigation in immediate continuity to the first registration. To achieve this the rabbit was taken away from the camera, repositioned, and a new accumulation of counts was made, followed by new area mapping and measurement. This investigation included 14 observations.

Each collection of counts took about 15 min including placing of the object, directing the gamma-camera and collection of data. The activity at the second determination was somewhat lower. This means that the collection time was longer, but had no influence on the compared results.

RESULTS

One rabbit fractured the leg and two died during the anaesthesia for roentgenographic examination leaving for evaluation eight rabbits.

Roentgenographic examination

Roentgenographic examination gave morphological information concerning bone formation but no quantitative data.

Three months postoperatively there was a bridging bone formation on the experimental side in six radius defects. In two defects there was only callus formation at the sawn ends. The central part was radiolucent indicating no mineral deposition. In these two cases there were no healing neither on the

grafted side nor on the control side. In the skin tube the implanted matrix showed mineral deposition though less than in the foreleg defect on the experimental side. On the control side, with no matrix implanted, there was a narrowing callus formation at the sawn ends in some cases reaching almost into the central part of the defect. In two rabbits there was a bridging mineralisation taking up most of the defect, fulfilling the criteria of healing. On the grafted side, however, these rabbits had even more bone formation.

At six months, radiographs showed almost the same picture. On the experimental side a further structure adaptation was noticed. There was cortical outlining and a radiolucent central part indicating a marrow component in six defects out of eight (Fig. 3). In the two defects, with no bridging bone formation, there was a reduction and rounding of callus formation compared to the observations at three months. In the skin tube there was almost the same radiopacity as shown in radiographs taken at three months postoperatively (Fig. 4). On the control side, in the six defects without bone filling the defect, no further bone formation was noticed compared to the observations at three months (Fig. 5).

Scintigraphy

At three months postoperatively the scintigraphy values on the grafted side in most cases showed one broad peak corresponding to an even bone formation within the matrix filling the defect. The control side with no graft usually showed two peaks with increased activity corresponding to the resection ends of the bone. At six months postoperatively the

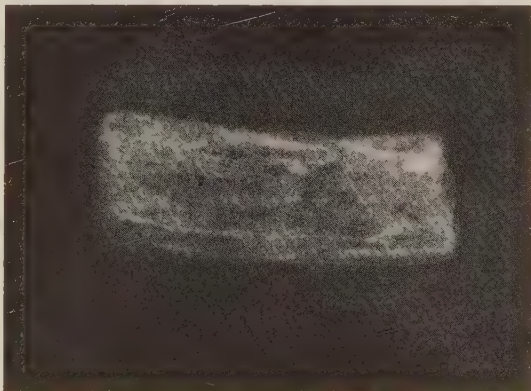


Fig. 4. The grafted skin tube 6 months postoperatively.

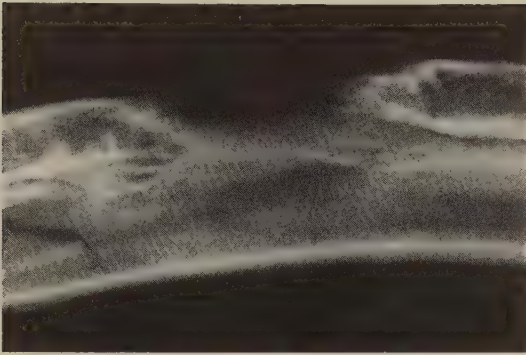


Fig. 5. The radial defect without graft 6 months postoperatively.

difference in activity had ceased (Fig. 2). The defect retransplanted with decalcified bone matrix thus, had a significantly ($p<0.01$) higher bone formation rate at three months postoperatively with a mean increase of 47% compared to the control side, whereas the difference had ceased at six months postoperatively (Table I). In the skin tubes somewhat increased activity was registered corresponding to the implanted pieces of bone matrix, but no quantitation was performed.

The error or method for the quantitative scintigraphy determined by measuring the relative counts accumulated during two consecutive occasions within identical areas was calculated to 10.2% (Table II).

DISCUSSION

To evaluate the metabolic activity within bone, radioactive bone seeking pharmaceuticals have been widely used. ⁹⁹Tc^m labelled phosphates and various other phosphonate compounds (⁹⁹Tc^mMDP, ⁹⁹Tc^m EHDP) as well as ¹⁸F, ⁸⁵Sr and ⁴⁵Ca accumulate within areas with increased bone metabolism (Weber, Keyes, Wilson & Landman, 1976).

Table I. Relative counts of scintigraphy determination at three and six months postoperatively in the radial defects

p-value according to Wilcoxon matched pairs signed-ranks test. Rabbit no. 6 died during anaesthesia for X-ray examination

Rabbit no.	3 months		6 months	
	Left	Right	Left	Right
1	6.6	4.9	4.9	4.3
2	11.4	3.9	4.9	3.9
3	6.7	5.8	0.8	4.8
4	14.3	9.7	5.6	8.2
5	16.1	14.7	7.6	10.0
6	18.6	12.5		
7	19.2	15.5	5.6	7.6
8	28.3	15.6	5.7	2.0
Mean	15.15	10.33	5.01	5.83
	<i>p</i> <0.01		N.S.	

Factors which influence the actual local uptake of these radiopharmaceuticals are blood flow, the capillary permeability, the bone mass, the exchangeable bone pool and the bone metabolism (King, Klipper & Weber, 1977). The relative importance of the different parameters is not quite evident. The local blood flow and the volume of accessible active metabolic bone have, however, shown the greatest measurable effect on the uptake (King et al.).

In the present investigation adult rabbits were used under standardized conditions, i.e. the volume of investigated bone mass both within and between animals may be considered equal. The same operation method has been used, which gives an equally randomized influence on the area both considering blood flow and increase in volume through callus formation at the sawn bone ends as well as the possible changes in vascular permeability and increase of extra-cellular edema caused by the operation trauma. The inflammatory reaction and possi-

Table II. Results of double determinations of activity ratios within identical areas of the defects

Determination														
I	0.8	4.8	5.2	12.1	12.6	6.3	9.9	16.1	14.7	7.7	18.5	17.5	5.0	7.8
II	1.8	4.3	3.2	14.3	9.7	5.6	8.2	16.6	15.4	7.4	19.2	15.5	5.6	7.6
Mean	9.76													
S.D.	0.99													
V %	10.2													

ble infection might also vary within the individual. After three months when the first measurement was performed this ought to have ceased and not influence the uptake of the bone-seeking radionuclide systematically in any direction. Repositioning of the gamma-camera, the orientation of the animal on the investigation table as well as small variations in measuring time had no significant influence on the results as shown by determination of the error of method in the present study.

A gamma-camera with pinhole collimator was used to measure the γ -radiation activity. The pinhole collimator consists of a coned lead mantle facing the object with a variable opening by a diaphragm insertion. In these studies a diaphragm with 6 mm diameter has been used. A smaller opening would have given less distortion in the picture and an increase in the resolution. With a smaller diaphragm insertion, however, the picture area is decreased which makes structure identification more difficult. The distance from the upper surface of the object to the collimator opening in this study was 65 mm which allowed a picture area including the radius, the ulna, the elbow and most of the humerus.

The predominant opinion is that $^{99}\text{Tc}^{\text{m}}$ -compounds react with the inorganic part of the bone tissue by sorption onto the hydroxyapatite crystals (Subramanian, McAfee, Bell, Blair, O'Mara & Ralston, 1972). The possibility has also been put forward that the addition depends on an affinity for the organic matrix component (Kaye, Silverton & Rosenthal, 1975; Rosenthal & Kaye 1975). The immature collagen in the bone-forming process would then be accessible for the $^{99}\text{Tc}^{\text{m}}$ -compound. According to Urist the reimplanted decalcified matrix is an inductor for remineralisation through mesenchymal cells differentiating into hypertrophied cells competent to synthesize collagen as a new framework for mineralisation. Before precipitation of hydroxyapatite crystals there is a zone of newly formed immature collagen which could adsorb the $^{99}\text{Tc}^{\text{m}}$ -compound. Whether this compound reacts with the bone matrix before mineralisation or later in the process of precipitation of the hydroxyapatite crystals does not have any influence on the results in this experimental model. In any case $^{99}\text{Tc}^{\text{m}}$ is a measure of the bone forming activity. Furthermore, studies on the distribution of $^{99}\text{Tc}^{\text{m}}$ -phosphate compounds in osteoarthritic femoral heads favour the possibility of deposition to the

hydroxyapatite crystals (Christensen & Arnoldi, 1980).

The present study has shown that the radionuclide uptake decreased from three months to six months postoperatively in spite of an increase in bone mass verified by radiographs and also by microscopy (to be published). If deposition were only a function of surface area, and not related to the metabolic activity, the matrix side in the six months investigation would give an uptake at least as great as the control side and would not show any change in the difference between ratios for the three months and six months (Table I).

The activity at 6 months on both the experimental and the control side were almost the same (Table I) and the ratios around one. This indicates that the mineralisation process has ceased and no additional bone-forming activity in the defects would be expected.

In conclusion autogenous decalcified bone matrix increases the bone formation after resection giving early restoration of the configuration when implanted in a created bone defect. Furthermore, technetium diphosphonate scintigraphy was shown to be an advantageous method to sequentially follow the rate of bone formation even in experiments with relatively small measuring areas. For early evaluation of the bone formation the scintigraphy was found to be superior to roentgenographic examination giving direct quantified information concerning the bone formation.

REFERENCES

- Albrektsson, T., Brånemark, P. -I., Hansson, H. -A. & Lindström, J. 1981. Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone-to-implant anchorage in man. *Acta Orthop Scand* 52, 155.
- Austermann, K. H., Becker, R., Büning, K. & Machten, E. 1977. Titanium implants as a temporary replacement of mandible. *J Max Fac Surg* 5, 167.
- Brånemark, P. -I., Breine, U., Adell, R., Hansson, B. -O., Lindström, J. & Ohlsson, Å. 1969. Intraosseous anchorage of dental prostheses. 1. Experimental studies. *Scand J Plast Reconstr Surg* 3, 81.
- Burchardt, H., Glowzewske, F. P. & Enneking, W. F. 1977. Allogenic segmental fibular transplants in azathioprine immunosuppressed dogs. *J Bone Joint Surg* 59-A 7, 881.
- Buring, K. 1974. Studies on bone induction. Thesis.
- Burwell, R. G., Gowland, G. & Dexter, F. 1963. Studies in the transplantation of bone. VI. Further observations concerning the antigenicity of homologous cortical and cancellous bone. *J Bone Joint Surg* 45-B, 597.

- Chalmers, J. 1959. Transplantation immunity in bone homografting. *J Bone Joint Surg (Br)* 41-B, 160.
- Christensen, S. B. & Arnoldi, C. C. 1980. Distribution of ^{99m}Tc -phosphate compounds in osteoarthritic femoral heads. *J Bone Joint Surg* 62-A, 90.
- Elves, M. W. 1974. *Int Arch Allergy Appl Immunol* 47, 708.
- Emmings, F. G., Neiders, M. E., Greene, G. W., Jr, Sheldon, W. K. & Gage A. A. 1966. Freezing the mandible without excision. *J Oral Surg* 24, 145.
- Gage, A. A., Greene, G. W., Jr, Neiders, M. E. & Emmings, F. G. 1966. Freezing bone without excision: an experimental study of bone-cell destruction and regrowth. *JAMA* 196, 90.
- Kaye, M., Silvertown, S. & Rosenthal, L. 1975. Technetium-99m-pyrophosphate. Studies in vivo and in vitro. *J Nucl Med* 16, 40.
- King, M. A., Klipper, R. W. & Weber, D. A. 1977. A model for local accumulation of bone imaging radiopharmaceuticals. *J Nucl Med* 18, 1106.
- Kreuz, F. P., Hyatt, G. W., Turner, T. C. & Bassett, A. L. 1951. The preservation and clinical use of freeze-dried bone. *J Bone Joint Surg* 33-A, 863.
- Lindström, J., Brånemark, P. -I. & Albrektsson, T. 1981. Mandibular reconstruction using the preformed autologous bone graft. *Scand J Plast Reconstr Surg* (in press).
- Marchetta, F. C., Sako, K., Murphy, I. B. 1971. The periosteum of the mandible and intraoral carcinoma. *Am J Surg* 122, 711.
- Marciani, R. D., Giansanti, J. S. & Massey, G. B. 1967. Reimplantation of freeze-treated and saline-treated mandibular bone. *J Oral Surg* 34, 314.
- Marcove, R. C. & Miller, T. R. 1969. Treatment of primary and metastatic bone tumors by cryosurgery. *JAMA* 207, 1890.
- Muscolo, D. L., Kawai, S. & Roy, R. D. 1977. In vitro studies of transplantation antigens in the rat. *J Bone Joint Surg* 59-B, 510.
- Muscolo, D. L. & Kawai, S. 1977. Bone transplantation antigens. Cellular and humoral immune response studies. *International orthopaedics (SICOT)* 1, 5. Springer-Verlag.
- Osborne, D. B., Lilly, G. E., Thompson, C. W. & Jost, T. 1977. Bone grafts with surface decalcified allogenic and particular autologous bone: report of cases. *J Oral Surg* 35, 276.
- Parrish, F. F. 1973. Allograft replacement of all or part of the end of a long bone following excision of a tumor. Report of twenty-one cases. *J Bone Joint Surg* 55-A, 1.
- Rosenthal, L. & Kaye, M. 1975. Technetium-99m-pyrophosphate kinetics and imaging in metabolic bone disease. *J Nucl Med* 16, 33.
- Scheunemann, H. 1976. Zur sekundären Osteoplastik nach temporärer Kinnrekonstruktion mit Silastik im Jugendlichen Alter. *Fortschr Kiefer Gesichtschir Band XX*, 42. George Thieme Verlag.
- Schmoker, R., Spiessl, B. & Mathys, R. 1976. A total mandibular plate to bridge large defects of the mandible. *New Concepts in Maxillofacial Bone Surgery*. Springer-Verlag.
- Smith, D. B. & Weaver, A. W. 1976. Cryosurgery for oral cancer, a six year retrospective study. *J Oral Surg* 34 (3), 245.
- Subramanian, G., McAfee, J. G., Bell, E. G., Blair, R. J., O'Mara, R. E. & Ralston, P. H. 1972. ^{99m}Tc -labeled polyphosphate as a skeletal imaging agent. *Radiology* 102, 701.
- Urist, M. R. 1970. The substratum for bone morphogenesis. *Dev Biol, Suppl.* 4, 125.
- Weber, D. A., Keyes, J. W., Wilson, G. A. & Landman, S. 1976. Kinetics and imaging characteristics of ^{99m}Tc -labeled complexes used for bone imaging. *Radiology* 120, 615.
- Williams, G. 1964. Experiences with boiled cadaveric cancellous bone for fracture of long bones. *J Bone Joint Surg* 46, 398.

Osteogenetic Activity in Composite Grafts of Demineralized Compact Bone and Marrow

JAN WITTBJER, D.D.S.,* BJÖRN PALMER, M.D.,** MADELEINE ROHLIN, D.D.S., PH.D.,†
AND KARL-GÖRAN THORNGREN, M.D.‡

The presence of osteogenic precursor cells in marrow has been extensively evaluated.^{1,4,10} Bone marrow is composed of myelogenous and stromal cells,⁶ which, although integrated in the marrow tissue, are morphologically and functionally different and extremely difficult to separate from each other.¹ In bone marrow transplants, hemopoietic cells die shortly after transplantation, and there is an overgrowth of fibroblasts.¹ These connective tissue elements are reversible modulations of one and the same cell type, a stem cell.² They can dedifferentiate and still have an ability to proliferate, redifferentiate, and give rise to bone formation.^{1,6,9}

Methods to improve osteogenesis by adding fresh autogeneic marrow, especially to allografts, have been the subject of several

experimental studies.^{3,12} Burwell³ and Nade and Burwell⁸ claimed that autogeneic fresh bone marrow is a useful contributor to a graft of demineralized allogeneic iliac bone. According to these investigators, demineralized bone acts only as a framework for mineralization, and the transplanted marrow cells are essential for the osteogenesis. This gives rise to the question of the extent to which transplanted fresh marrow cells contribute to new bone formation in a bone defect when combined with demineralized compact autogeneic bone.

The difference in osteogenetic activity between demineralized autogeneic compact bone compared with a composite graft in which fresh autogeneic marrow was added to demineralized autogeneic compact bone was analyzed.

MATERIALS AND METHODS

In 30 adult rabbits with an average body weight of 3.1 kg, a piece of the radius was bilaterally resected under Mebumal anesthesia (ACO, Solna, Sweden) supplemented with local anesthesia (Xylocain®, 0.5%; Astra, Södertälje, Sweden).

At a second operation, after demineralization, the resected pieces of bone, *i.e.*, bone matrix, were replaced in the radial defects. On one side, bone matrix was supplemented with autogeneic marrow. The rabbits were killed 14 or 28 days after operation. The remineralization process was then evaluated by roentgenography, including planimetry, scintigraphy, and autoradiography.

* Maxillofacial Center, Department of Nuclear Medicine, Malmö General Hospital and Department of Prosthetic Dentistry, School of Dentistry, University of Lund, Sweden.

** Department of Plastic Surgery, Malmö General Hospital, Malmö, Sweden.

† Department of Oral Radiology, School of Dentistry, University of Lund, Sweden.

‡ Department of Orthopaedic Surgery, Lund University Hospital, Lund, Sweden.

Supported by the Faculties of Odontology and Medicine, University of Lund, by Greta and Johan Kocks Foundations, and by the Swedish Medical Research Council (Project 17X-2031).

Reprint requests to Jan Wittbjer, D.D.S., Maxillofacial Center, Malmö General Hospital, S-214 01 Malmö, Sweden.

Received: April 5, 1982.

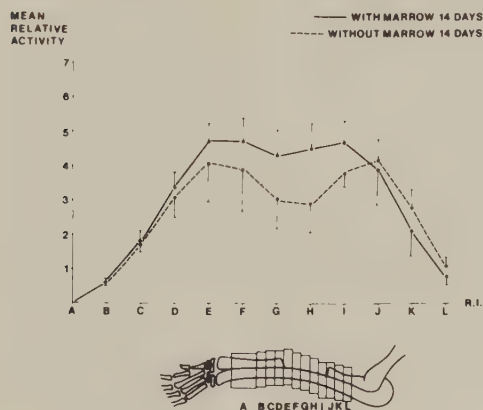


FIG. 1A. Scintigraphic examination. Mean relative counts $\pm$ SEM to the control area (A) in regions of interest (RI) 14 days after operation on the experimental side (with marrow) and control side (without marrow).

SURGICAL PROCEDURE

A 12-mm piece of the radius, including periosteum, was resected during continuous irrigation with saline. Sterile plastic tubing (Nelaton® catheter Steritex A/S, Denmark), with dimensions corresponding to those of the resected bone, was placed in the defect. The two resected bone pieces were rinsed of periosteum and bone marrow, demineralized in 0.6 N HCl for 24 hours, rinsed

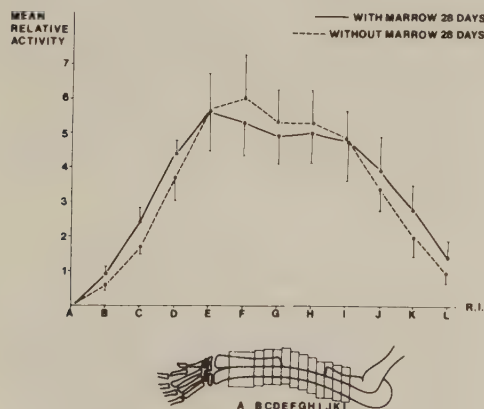


FIG. 1B. Scintigraphic examination. Mean relative activity $\pm$ SEM to the control area (A) in regions of interest (RI) 28 days after operation on the experimental side (with marrow) and control side (without marrow).

again, and kept in saline at 4° for two days. Three days after the first operation, the wounds were bilaterally opened and the plastic tubing removed. On one side, alternating right and left sides, only the demineralized bone (matrix) was reimplanted (control side). On the other side, matrix and marrow were placed in the defect (experimental side). The bone marrow (0.2 ml) was obtained by aspiration with a syringe and nonpyrogenic plastic tubing (Butterfly-23®, Abbot Ireland Ltd.) from the femoral medullary cavity through a drill hole and immediately administered in excess into the defect. The wounds were closed in anatomic planes.

ROENTGENOGRAPHY

After the rabbits were killed, the forelegs were removed. The bone specimens were examined with a dental X-ray unit using Kodak Occlusal Ultra-speed D films. Exposure time was 0.4 second. A subjective evaluation of the roentgenograms was carried out by one of the authors twice, in the form of a blind-test. The roentgenograms from the left and right sides of each rabbit were compared, and that showing the most extensive mineralization was selected. The roentgenograms also were placed in an X-ray film enlarger/viewer (Realist Inc. Photographic Products Division, Menomonee Falls, Wisconsin) with an optic magnification of 10:1. The display was covered with transparent paper on which the outlines of the mineralized tissue in the bone defects were drawn. Thereafter, the defects were estimated with a planimeter (Los Angeles Scientific Instruments Co., Inc.). The extension of mineralization was expressed as percentage of the area of the whole bone defect.

SCINTIGRAPHY

Technetium-99m-labelled methylenediphosphonate (^{99m}Tc -MDP) was prepared by adding ^{99m}Tc -pertechnetate (AB Atomenergi, Studsvik, Sweden) to an MDP-kit (Kabi Diagnostica, Sweden) containing 5.00 mg of MDP and 0.84 mg of SnCl_2 . After addition of pertechnetate, the solution was diluted to 6 ml. From this solution, about 2 ml with an activity of about 180 MBq (≈ 5 mCi) (58 MBq/kg body weight) was given intravenously to the rabbits via an ear vein, three hours before scintigraphic examination.

A gamma-camera with pinhole collimator registered the emitted gamma radiation. The numeric data were stored in a disc memory. Regions of interest were mapped, covering an area including the displayed defect and the sites of resection (Figs.

1A and 1B). Mean counts in every region of interest were measured separately, and the differences to the control area (A in Fig. 1A) were calculated. Relative values to the control area were then calculated (Figs. 1A and 1B). The scintigraphic method, as well as the determination of the error of this method, is described in more detail in a previous study.¹⁶

AUTORADIOGRAPHY

Immediately after roentgenographic and scintigraphic examination, the right and left radii from eight animals chosen at random from each group were mounted on a microtome stage in carboxymethyl cellulose mixed with water. The stage was immersed in a mixture of hexane and solid CO₂ (-75°). Under refrigeration (-15°), sagittal sections were cut at five to ten different levels. To obtain whole sections 20 µm thick, an adhesive tape (No 471 3 M, Minnesota Mining and Manufacturing Corporation) was applied to the section surface of the frozen specimen before cutting.

Autoradiographic examination was performed by apposition of the sections and the frozen block containing the remainder of the legs against Structurix D7 films (Agfa-Gevaert, Antwerpen, Belgium). Due to the short half-life of ^{99m}Tc (6 hours), time did not permit drying the sections before pressing them against the films. Thus, the sections and block were kept frozen, and the apposition occurred in a refrigerator (-15°). Sections, block, and films were kept under refrigeration during exposure. The exposure time was 72 hours. After exposure the sections and block were separated from the films and the sections allowed to freeze-dry at -15°. The films were developed with Gevaert G 138 for about two minutes (+20°) and fixed with Gevaert G 334 for ten minutes. The technique for freeze-sectioning and autoradiography has been described in detail by Ullberg.¹³ The autoradiographic technique for ^{99m}Tc-labelled sections has previously been described by Rohlin and Hammarström.¹¹

RESULTS

One rabbit suffered a fractured leg, and two died during anesthesia for the second operation, leaving for roentgenographic examination 13 in the 14-day group and 14 in the 28-day group. Five rabbits in the 28-day group received ^{99m}Tc-pertechnetate instead of ^{99m}Tc-MDP and were excluded from scintigraphic examination. Two were missing in

the computer memory, resulting in a scintigraphic registration containing 11 and nine rabbits, respectively.

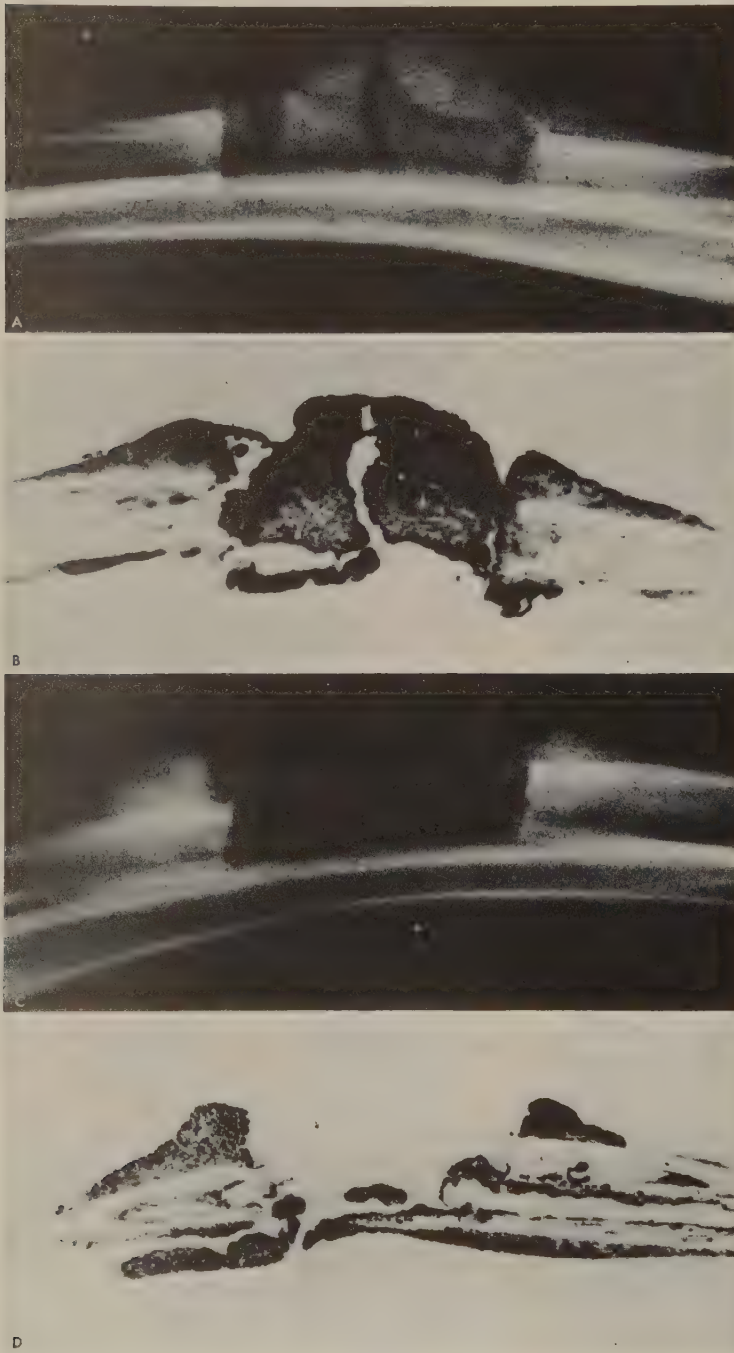
ROENTGENOGRAPHY

When planimetric measurement of mineralized areas resulted in a difference between experimental and control sides of 3% or less, the extension of mineralization was judged to be equal on the two sides. The two subjective evaluations were in accordance with each other, and under circumstances described above, were in accordance with the planimetric evaluations in all but one rabbit, Rabbit 25, which was judged equal at the subjective evaluation. At the outside of the sites of resection, a triangular radiopacity, representing callus formation, was observed in all rabbits (Figs. 2A, 2C, 3A and 3C).

Fourteen days after operation, radiopacity, indicating mineralization, partly filled the bone defect. More extended mineralization on the experimental (matrix + marrow) side (Fig. 2A) compared with the control (matrix) side (Fig. 2C) was observed in all but two rabbits. In these two rabbits (Rabbits 3 and 6), more bone tissue was observed on the control side. Generally, however, the planimetric estimation indicated a significantly more extended mineralized deposition ($p < 0.05$) on the experimental side (Table 1).

Twenty-eight days after operation, the extension of mineralized tissue inside the bone defect on the experimental side (Fig. 3A) was judged to be equal to that of the control side (Fig. 3C) in most rabbits (8 of 14). In the remaining six rabbits, four had a more widespread mineralization on the experimental side and two on the control side (Rabbits 21 and 22). The planimetric estimation showed the difference between the experimental and control sides to be nonsignificant (Table 1).

The areas of mineral deposition inside the bone defects were extensive, occupying about 90% of the total bone defect for both groups, representing an increase of 33% on the ex-



FIGS. 2A-2D. Roentgenographic and autoradiographic examination. Left and right radius and ulna from adult rabbit 14 days after operation. (A and C) Roentgenograms and (B and D) the corresponding autoradiograms 4 hours after intravenous injection of ^{99m}Tc -labelled MDP. (A and B) Experimental side with demineralized bone and marrow grafted to the bone defect. Mineralized tissue is present in nearly the whole defect. Newly mineralized tissue is also observed at the sites of resection. In the corresponding autoradiogram, uptake of ^{99m}Tc is visible at the outside of the sites of resection and in the majority of the bone defect. There is no uptake in the very small areas near the sites of resection or in the middle of the bone defect. The ulna is missing in this autoradiogram. (C and D) Control side with demineralized bone grafted to the bone defect. Newly mineralized tissue is only visible in triangular areas at the outside of the sites of resection and at the surface of the ulna. In the corresponding autoradiogram, there is a high uptake in described areas, while no uptake is visible inside the bone defect.

FIGS. 3A-3D. Left and right radius from adult rabbit 28 days after operation. (A and C) Roentgenograms in which the ulna is also included and (B and D) the corresponding autoradiograms 4 hours after intravenous injection of ^{99m}Tc -labelled MDP. (A and B) Radius in which demineralized bone and marrow were grafted to the bone defect. (C and D) Radius in which only demineralized bone was grafted to the bone defect. The whole bone defect is filled with newly mineralized tissue with the same opacity as that of the surrounding cancellous bone. Outside the sites of resection, there is a triangular thickening of bone tissue. In both corresponding autoradiograms there is a high uptake of ^{99m}Tc in the whole bone defect and in the triangular areas outside the radius. Only in small areas is there no uptake.

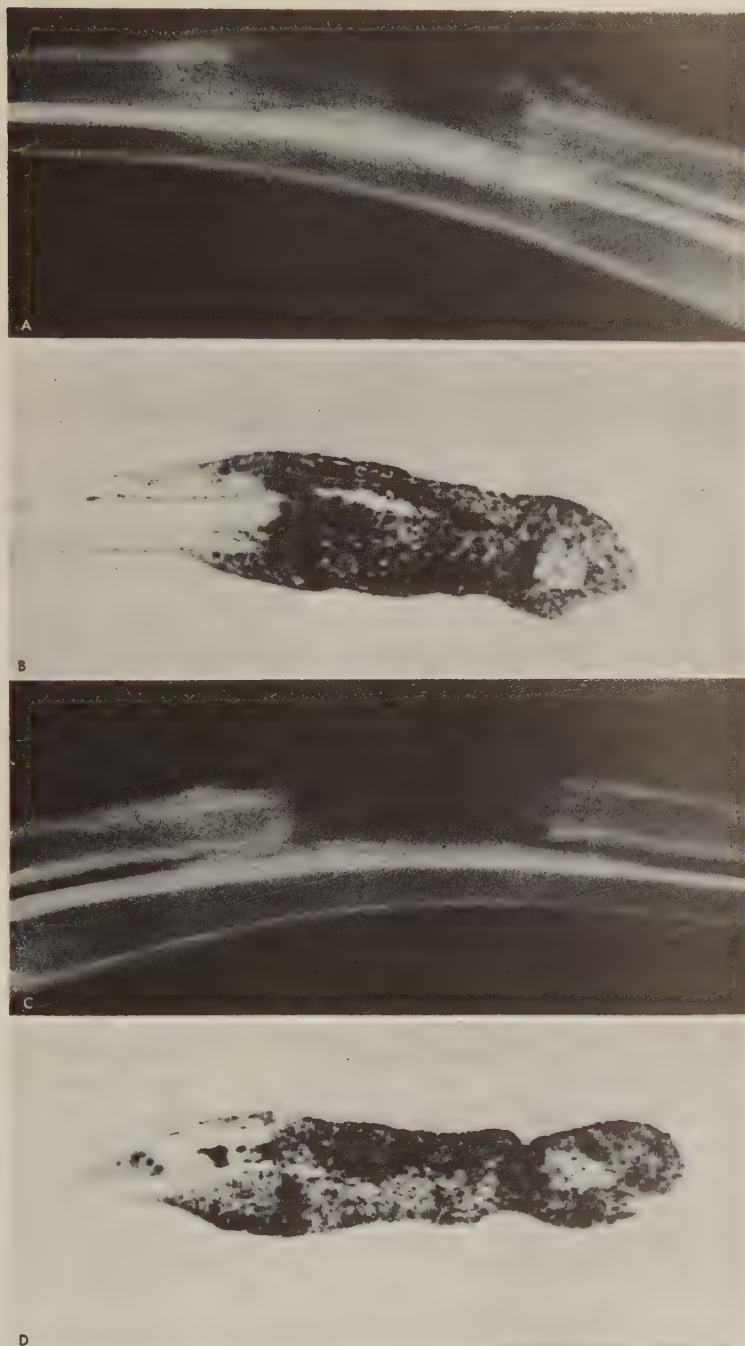


TABLE 1. Roentgenographic Examination: Planimetric Evaluation of Mineralized Areas in Percentage of the Radial Initial Bone Defect

<i>Rabbit No.</i> <i>(14-day group)</i>	<i>Matrix</i> <i>+ Marrow</i>	<i>Matrix</i>	<i>Rabbit No.</i> <i>(28-day group)</i>	<i>Matrix</i> <i>+ Marrow</i>	<i>Matrix</i>
1	80	50	14	98	94
2	93	65	15	91	89
3	43	86	16	94	97
4	88	77	17	99	98
5	53	37	18	98	86
6	23	47	19	93	95
7	79	46	20	100	99
8	85	42	21	83	91
9	23	17	22	78	83
10	74	31	23	87	82
11	94	35	24	77	66
12	67	42	25	87	81
13	90	45	26	89	90
			27	98	98
Mean	68.6	47.7		90.9	89.2
	$p < 0.05^*$			NS	

* p -values according to Wilcoxon's matched pairs signed-ranks test of differences between experimental side (matrix + marrow) and control side (matrix).

NS = not significant.

perimental side and 85% on the control side, in comparison with increases found 14 days after operation.

SCINTIGRAPHY

Fourteen days after operation, the scintigraphic values of the specimens on the experimental side mostly showed an almost even bone formation within the whole bone defect (Fig. 1A). On the control side, there were two peaks corresponding to the resection ends of the radius, while a significantly lower activity ($p < 0.01$) was registered in the central part of the bone defect (Table 2).

Twenty-eight days after operation, there was no significant difference between the two sides (Fig. 1B, Table 3). Generally, the activity was higher at 28 days compared with 14 days, irrespective of marrow addition (compare Fig. 1A with Fig. 1B).

AUTORADIOGRAPHY

Uptake of ^{99m}Tc was seen where new bone formation was observed roentgenographi-

cally (Figs. 2 and 3). However, the area of uptake was mostly smaller than the area indicating mineralization in the corresponding roentgenogram. In small, thin areas near the radial ends (Fig. 2B) and inside the graft, areas without uptake were often observed (Figs. 2B, 3B, and 3D). A triangular area of uptake corresponding to the site of callus formation at the sites of resection was seen in all radii (Figs. 2B, 2D, 3B, and 3D).

Fourteen days after operation, activity often was visible along the borders of the graft. A patchy uptake could be observed inside the defects. These areas of uptake were mostly larger on the experimental side (Figs. 2B and 2D).

Twenty-eight days after operation, there was a pronounced uptake in the whole defect on both sides in most animals, independent of supplemented marrow (Figs. 3B and 3D).

DISCUSSION

It is generally agreed that nondemineralized devitalized bone fails to produce more

TABLE 2. Scintigraphic Examination 14 Days after Operation: Relative Activity on Experimental (matrix + marrow) and Control Side (matrix) of the Radial Defects at the Center and at Sites of Resection (proximal, distal)

Rabbit No.	Matrix + Marrow			Matrix		
	Proximal	Center	Distal	Proximal	Center	Distal
1	3.2	2.9	3.5	3.4	1.8	2.5
2	3.3	2.7	3.2	4.1	3.7	3.7
3	4.6	4.4	5.7	4.3	4.1	5.2
4	2.6	3.2	4.1	2.8	2.7	2.8
5	6.6	4.5	4.4	7.3	3.7	6.0
6	5.0	8.8	6.8	4.1	5.6	4.7
7	3.6	3.5	5.4	4.2	2.8	4.7
8	10.3	9.6	8.6	8.1	3.0	6.0
9	6.7	3.9	4.2	4.2	2.9	4.5
12	4.4	1.9	2.7	3.8	1.0	2.7
13	3.5	2.8	3.3	4.9	1.0	2.6
Mean	4.89		4.72	4.65		4.13
		4.38	$p < 0.01^*$		2.94	

* p-value according to Wilcoxon's matched pairs signed-ranks test.

than sparse deposits of bone when implanted in a muscular site¹⁴ or in a bone defect.¹⁷ According to Nade and Burwell,⁸ demineralized cancellous bone only acts as a framework for mineralization when used in a com-

posite graft together with fresh autologous marrow. The inductive capacity differs, however, between demineralized compact bone and demineralized cancellous bone in favor of compact bone, probably owing to the

TABLE 3. Scintigraphic Examination 28 Days after Operation: Relative Activity on Experimental (matrix + marrow) and Control Side (matrix) of the Radial Defects at the Center and at Sites of Resection (proximal, distal)

Rabbit No.	Matrix + Marrow			Matrix		
	Proximal	Center	Distal	Proximal	Center	Distal
19	11.2	7.0	6.0	8.9	6.5	6.2
20	8.1	8.1	7.5	4.5	3.5	3.8
21	3.9	3.9	5.2	2.9	2.7	5.2
22	2.3	1.1	2.0	4.0	3.6	4.6
23	3.1	1.7	2.0	3.7	3.0	3.3
24	5.4	8.1	6.3	7.2	5.4	5.0
25	6.8	6.7	11.6	8.1	11.0	14.9
26	3.5	3.7	3.5	5.5	8.0	7.4
27	4.4	4.1	4.4	2.0	2.2	2.4
Mean	5.41		5.39	5.20		5.87
		4.93	$p = NS^*$		5.10	

* p-value according to Wilcoxon's matched pairs signed-ranks test.

NS = not significant.

sparse collagen structure in cancellous bone.¹⁴ The marrow was obtained by aspiration and applied to reach the packing density that is essential for marrow transplantation.⁵

The examination methods supplemented each other. Roentgenography reflected the morphologic product of mineralization after 14 and 28 days, respectively, in contrast to scintigraphy and autoradiography, which revealed the momentary activity of mineralization. Roentgenograms, as well as scintigrams, gave information on the total amount of mineralization inside the bone defect, whereas the autoradiograms presented the activity of only one plane of the graft. Thus, the areas of radioactivity shown in autoradiograms were smaller than the areas of mineralization observed in roentgenograms. To have a true and detailed picture of the mineralization throughout the whole bone defect, serial freeze-sectioning has to be performed.

At the subjective evaluations of the roentgenograms, it was possible to discriminate small differences in the extension of mineralization between the experimental and control sides. However, the figures from the planimetric evaluation, which were more time-consuming, were useful in expressing the difference, not only between the experimental and control sides, but also between the 14-day and 28-day groups.

To discriminate the activity in the graft from the activity caused by callus formation at the sites of resection of the radius, the scintigraphic determinations of the activity were expressed as mean counts in regions of interest covering the defect. The relative activity in the center of each bone defect and site of resection was then calculated separately, facilitating the comparison between the groups.

The significant difference in mineralization between the experimental and control sides in the bone defect 14 days after operation, shown both roentgenographically and

with ^{99m}Tc-labelled diphosphonate in this study, indicated the stimulatory effect of added marrow. However, this difference between the two groups had ceased 28 days after operation. The results from the study with ^{99m}Tc-labelled diphosphonate could indicate that the experimental side reached its peak for mineralization at 14 days and was declining at 28 days, whereas the control side reached the peak later, almost 28 days after operation. Conversely, the roentgenograms obtained 28 days compared with those obtained 14 days after operation showed a progressing mineralization that was more pronounced on the control side. Lindholm and Urist⁷ reported similar results with composite grafts of allogeneic bone matrix and autogeneic bone marrow implanted into an abdominal muscle pouch. According to their findings, the influence on bone formation by the marrow cells is greatest in the first generation, with their inherent osteogenic competence, whereas the induction from the matrix starts later, because the degradation of collagen structure to bone morphogenetic protein¹⁵ is delayed. This might explain the lack of difference 28 days after operation in uptake of ^{99m}Tc-labelled MDP between the two sides and the lack of difference in the total amount of bone mineralized. Another possibility requiring further investigation is the suggestion by Hirano and Urist⁶ that a bone marrow-derived matrix-resorbing cell population by some unknown mechanism could inhibit proliferation of cartilage and bone precursor cells and thereby reduce the osteogenic activity.

There are several clinical conditions in which reconstruction of a defect with autogeneic demineralized bone would be useful. Surgery of soft tissue tumors adjacent to the jaw that includes partial resection of the mandible is destructive and mutilating. For rehabilitation, sufficient restitution, both for function and cosmesis, is important. There are, however, great difficulties in finding suit-

able bone grafts and achieving incorporation of the graft in this area, as well as at some other locations in the body. Grafting resected bone after demineralization might be a solution, as it might serve as an inductive framework for reformation of bone under optimal conditions concerning the autogenicity and shape of the graft. Added autogeneic bone marrow could improve bone formation in the first important phase.

This technique of bone autografting could also be applicable in tumor surgery of the extremities, both when spacers of cement and metal are used after resection to achieve direct mobility during the period of cytostatic treatment, and when large bone resections are replaced by a long-stemmed, uncovered endoprosthesis. The unaffected pieces of bone could, after the specimen has been investigated for radicality, be demineralized and later retransplanted.

Bone autografting might also be applicable to complications after placement of long-stemmed knee endoprostheses. Arthrodesis is considered the best salvage procedure after a failed knee arthroplasty. Sufficient bone is, however, often lacking. Storage of the demineralized resected bone after any knee arthroplasty might be the patient's own reserve "bank bone," to be used if later needed.

In comminuted open contaminated fractures, pieces of bone have to be removed to prevent sequester formation. These bone pieces could, after demineralization, be reimplanted in connection with delayed skin-covering surgery. This procedure would decrease the amount of iliac autogeneic bone necessary for the total reconstruction of the bone defect.

In conclusion, bone otherwise discarded might be reused in combination with added bone marrow. There is evidence from the roentgenographic, scintigraphic, and autoradiographic investigations of the present study that vital marrow cells added to a demineralized autogeneic compact bone graft

are advantageous to the osteogenesis in the early stage of the healing process, while the later stage seems unaffected.

SUMMARY

The effects of a composite graft of autologous marrow and demineralized autologous compact bone on the healing of a surgically created bone defect were observed in adult rabbits. A segment of the radius was bilaterally resected, demineralized, and replaced. On one side the bone graft was supplemented with autologous marrow. The new bone formation was measured 14 and 28 days after operation by roentgenography, including planimetry with scintigraphy and autoradiography using ^{99m}Tc -labelled MDP. The composite graft, *i.e.*, demineralized compact bone and marrow, had a significantly higher ($p < 0.01$) bone formation rate 14 days after operation compared with the graft with demineralized compact bone in the opposite radius. At 28 days, however, there were no differences between the sides. Viable autologous marrow cells and demineralized autologous compact bone graft accelerate the rate of osteogenesis, but only at the beginning of the healing process.

REFERENCES

1. Ashton, B. A., Allen, T. D., Howlett, C. R., Eaglesom, C. C., Hattori, A., and Owen, M.: Formation of bone and cartilage by marrow stromal cells in diffusion chambers *in vivo*. *Clin. Orthop.* 151:294, 1980.
2. Bloom, W., Bloom, M. A., and McLean, F.: Calcification and ossification. *Anat. Rec.* 81:443, 1941.
3. Burwell, R. C.: Studies in the transplantation of bone. VII. The fresh composite homograft-autograft of cancellous bone: An analysis of factors leading to osteogenesis in marrow transplants and in marrow-containing bone grafts. *J. Bone Joint Surg.* 46B:110, 1964.
4. Friedenstein, A. J.: Determined and inducible osteogenetic precursor cells. *In* Hard Tissue, Ciba Symposium 11. Amsterdam, Assoc. Scientific Publ., 1969.
5. Friedenstein, A. J., Piatetzky-Shairo, I. I., and Petrakova, K. V.: Osteogenesis in transplants of bone

- marrow cells. *J. Embryol. Exp. Morph.* 16:381, 1966.
6. Hirano, H., and Urist, M. R.: Bone-forming and bone-resorbing cell lines derived from bone marrow in tissue culture. *Clin. Orthop.* 154:234, 1981.
 7. Lindholm, T. S., and Urist, M. R.: A quantitative analysis of new bone formation by induction in composite grafts of bone marrow and bone matrix. *Clin. Orthop.* 150:288, 1980.
 8. Nade, S., and Burwell, R. G.: Decalcified bone as a substrate for osteogenesis. An appraisal of the interrelation of bone and marrow in combined grafts. *J. Bone Joint Surg.* 59B:189, 1977.
 9. Patt, H. M., and Maloney, M.: Bone formation and resorption as a requirement for marrow development. *Proc. Soc. Exp. Biol. Med.* 140:205, 1972.
 10. Pfeiffer, C. A.: Development of bone from transplanted bone marrow in mice. *Anat. Rec.* 102:225, 1948.
 11. Rohlin, M., and Hammarström, L.: Whole-body autoradiography of $^{99}\text{Tc}^m$ -labelled pyrophosphate and related compounds in young rats. *Acta Radiol. (Ther.)* 15:71, 1976.
 12. Salama, R., Burwell, R. G., and Dickson, I. R.: Recombined grafts of bone and marrow. *J. Bone Joint Surg.* 55B:4002, 1973.
 13. Ullberg, S.: Studies on the distribution and fate of S^{35} -labelled benzyl-penicillin in the body. *Acta Radiol. (Suppl.)* 116, 1954.
 14. Urist, M. G.: Substratum for bone morphogenesis. *Dev. Biol.* 4(Suppl.):125, 1970.
 15. Urist, M. R., Mikulski, A. J., and Lietze, A.: Solubilized and insolubilized bone morphogenetic protein. *Proc. Natl. Acad. Sci.* 76:1828, 1979.
 16. Wittbjer, J., Nosslin, B., Palmer, B., and Thorngren, K.-G.: Bone formation of transplanted autologous bone matrix in rabbit evaluated by technetium radionuclide bone imaging. *Scand. J. Plast. Reconstr. Surg.* 16:23, 1982.
 17. Wittbjer, J., Palmer, B., and Thorngren, K.-G.: Osteogenetic properties of reimplanted decalcified and undecalcified autologous bone in the rabbit radius. *Scand. J. Plast. Reconstr. Surg.* 16:279, 1982.

BONE FORMATION IN DEMINERALIZED BONE TRANSPLANTS
TREATED WITH BIOSYNTHETIC HUMAN GROWTH HORMONE

J. Wittbjer, M. Rohlin and K.-G. Thorngren

From the Maxillofacial Centre, Departments of Plastic Surgery
and Nuclear Medicine, Malmö General Hospital and Departments
of Prosthetic Dentistry, Oral Radiology and Orthopaedic Surgery,
University of Lund, Sweden

Address: Dr. Jan Wittbjer, Maxillofacial Centre, Malmö General
Hospital, S-214 21 Malmö, Sweden

Supported by the Faculties of Odontology and Medicine,
University of Lund, by Kabi-Vitrium AB, Stockholm, and
by the Swedish Medical Research Council (Project No. 17X-2031).

ABSTRACT

The influence of local administration of biosynthetic human growth hormone on the bone formation in a rabbit bone defect grafted with demineralized autologous bone was studied and compared to contralateral saline administration. The growth hormone was produced by hybrid DNA-technique and administered locally to the bone defect with the demineralized bone implant by an osmotic pump delivering $5 \mu\text{l/hr}$ during 14 days. At 14 and 28 days after operation the bone forming process was evaluated by roentgenography, planimetry included, and scintigraphy after injection of $^{99}\text{Tc}^{\text{m}}$ -labelled DPD. At 28 days after operation also autoradiography was performed. The roentgenographic and scintigraphic examinations revealed no influence from growth hormone neither 14 nor 28 days postoperatively. Centrally in the remineralized grafts the roentgenograms 28 days after operation showed radiolucent areas which in corresponding autoradiograms showed no uptake of $^{99}\text{Tc}^{\text{m}}$. These areas turned out to be remnants of matrix. It is suggested that growth hormone has no influence on the bone induction process during the first 14 days when administered to bone matrix, probably due to relative lack of osteogenic cells. This study contributes a base for further evaluation of factors pertinent to hormonal stimulation of bone matrix transplants.

INTRODUCTION

The treatment of slow healing fractures and pseudoarthroses and the restitution of hard tissue defects are clinical problems in which bone grafting is essential. The best results are achieved with fresh autologous bone, but when a suitable autograft is not available substitutes have been used (Friedlaender 1982). Demineralized compact bone (Urist 1970) has an ability to act as a free graft stimulating new bone formation and bridging large bone defects (Wittbjer, Nosslin, Palmer & Thorngren, 1982). A substance that further enhances bone formation in a bone graft would be of great clinical importance.

Systemic administration of growth hormone regulates the proliferation and differentiation of precursor cells of bone (Reddi & Sullivan, 1980) and has been shown to have an accelerating effect on the osteoblastic activity (Handelsman & Gordon, 1930; Frost, 1962; Harris, Heaney, Jowsey, Cockin, Akins, Graham & Weinberg, 1972). The effect of growth hormone on longitudinal bone growth and periosteal bone remodelling in growing individuals is well known (Thorngren, 1973; Stenström, Hansson & Thorngren, 1976) whereas in adults the effect is under discussion. Both positive (Mankin, Thrasher, Weinberg & Harris, 1978; Koskinen, Lindholm, Nieminen, Puranen & Attila, 1978; Ahl & Kalén, 1979) and negative (Lindholm, Koskinen, Puranen, Nieminen, Kairaluoma & Attila, 1977; Harris, Bean & Banks, 1975; Northmore-Ball, Wood & Meggitt, 1980) results have been published concerning growth hormone and its influence on bone formation. The effect seems to depend on the size and age of the bone defect. In these studies systemic administration of growth hormone has been used, necessitating larger amounts. Recently,

however, Isacsson, Jansson & Gause (1982) have shown direct effects on the longitudinal bone growth process also by locally administered human growth hormone to the cartilage growth plate of hypophysectomized rats. Also Edén, Isaksson, Madsen & Friberg (1983) have recently found specific bindings of growth hormone to isolated chondrocytes from rabbit ear and epiphyseal growth plate which supports the concept of a direct effect of growth hormone. Consequently, local treatment of growth hormone would act directly on the target tissue, and thereby influence the metabolic activity of bone precursor cells which, according to Urist (1970), is generated by the osteogenic properties of implanted bone matrix.

The lack of human growth hormone has till now limited the possibilities for treatment and for experimental studies as well. It is now possible to produce growth hormone biosynthetically with gene modification techniques making larger quantities available for use in fields of new indications. The aim of this investigation was to study the effect of locally administered biosynthetic human growth hormone on the healing process of autologous demineralized bone grafts implanted in bone defects of the rabbit radii.

MATERIAL AND METHOD

General outline

Fifteen adult rabbits with an average body weight of 3.5 kg were operated on under anaesthesia consisting of intramuscular injection of Hypnorm Vet[®] (LEO, Helsingborg, Sweden) supplemented with intravenous

injection of Mebumal (ACO, Solna, Sweden) and local anaesthesia (Xylocain® 0.5%, Astra, Södertälje, Sweden). At a first operation a 12 mm piece of the radius was resected bilaterally and demineralized in 0.6 N HCl for 24 hours. After thoroughly rinsing in saline the bone pieces were kept at +4°C for two days. At a second operation the demineralized bone pieces were reimplanted in the defects. The method of operation is described in detail by Wittbjer, Nosslin, Palmer & Thorngren (1982). Biosynthetic somatotropin was administered locally in one defect, alternating right or left, with an osmotic pump. On the opposite side serving as a control side another osmotic pump filled with saline was used. The remineralization process was evaluated by roentgenography, scintigraphy and autoradiography.

Biosynthetic somatotropin

Biosynthetic human growth hormone (HGH, Kabi-Vitrum AB, Stockholm, Sweden) produced by hybride DNA-technique was used. This growth hormone preparation contains 2 IU/mg. The growth hormone was dissolved in saline. A total dose of 2.4 IU was administered to each rabbit locally.

Osmotic pump

The osmotic pump (Alzet® osmotic pump, Model 2 ML2, ALZA Corporation, Palo Alto, U.S.) used in this experiment has been developed for delivering a specified volume of 5 µl/hr during 14 days. These data guaranteed by the manufacturer are related to tests of pumping rate and volume determined in isotonic saline at +37°C. One pump was filled with approximately 2 ml growth hormone solution. At the top of the pump a silastic catheter filled with the solution was attached to a tube extension. The other pump was filled with approximately 2 ml saline.

After incision a subcutaneous pocket was made in the scapular region into which the pumps were inserted (Fig. 1). The catheters were tunnelled down to the paw leaving the opening over the reimplanted bone matrix and held in place by sutures. At sacrifice the catheters were controlled to be in correct position and the pumps to contain a small residue of somatotropin or saline, respectively.

Roentgenography

The bones were examined with a dental X-ray unit (Oralix 65, N.V. Philips, Eindhoven, Netherlands) using Kodak Occlusal Ultra-Speed D films and an exposure time of 0.4 second. During the roentgenographic and scintigraphic examination 14 days after operation the rabbit was anesthetized with Hypnorm Vet[®]. Twenty-eight days after the operation the right and left foreleg specimens were examined placed on the same X-ray film. The resulting roentgenograms were placed in an X-ray film enlarger/viewer (Realist Inc. Photographic Products Division, Wisconsin, U.S.A.) with an optic magnification of 10:1. The display was covered with transparent paper on which the outlines of the bone defect and the mineralized tissue inside the bone defect were drawn. The remineralized area was then estimated with a planimeter (Los Angeles Scientific Instruments Co., Los Angeles, U.S.A.). The extent of the remineralization was expressed in percentage of the radial initial bone defect (Table I). The extent of mineralization was judged to be equal on the two sides when there was a difference of three per cent or less.

Scintigraphy

$^{99}\text{Tc}^{\text{m}}$ -DPD, a technetium-99m labelled 3,3 diphosphono - 1,2 - propanedicarboxylic acid, tetra sodium salt (Behringwerke AG, Marburg,

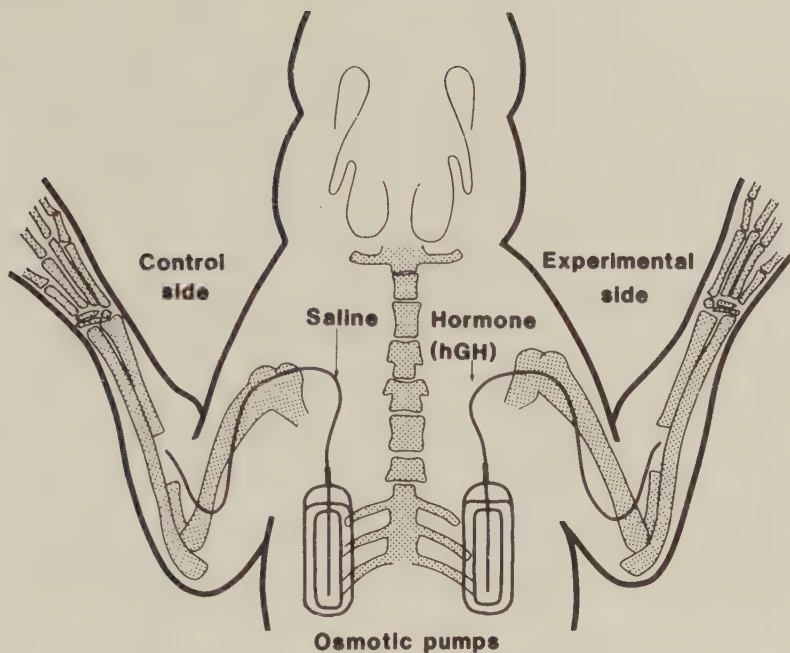


Fig. 1. Implanted osmotic pumps with catheters leading to the radial defects.

Table I. Roentgenographic examination: Planimetric evaluation of mineralized areas in percentage of the radial initial bone defect

Rabbit no.	14 days		28 days	
	Hormone	Saline	Hormone	Saline
1	10	28	41	97
2	15	68	96	98
3	94	79	99	100
4	30	26	95	90
5	34	10	97	94
6	25	13	x	x
7	12	19	x	x
8	29	26	98	91
9	77	66	94	94
10	63	72	84	95
11	73	42	95	97
Mean	42	41	89	95

x = inadequate technical quality

West Germany) was injected intravenously three hours before the animals were killed. The injected solution contained an activity of approximately 350 MBq. The gamma radiation was registered with a gamma-camera. Mean counts were calculated in regions of interest mapped out covering the defect area and sites of resection. Relative values to a control area (A in Figs 5, 6) outside the defect were then calculated. The mean values in every region as well as the relative values in the centre and in the proximal and distal part of the defect at both sides were calculated (Table II, Figs 5, 6). The method has previously been described in more detail (Wittbjer, Nosslin, Palmer & Thorngren, 1982).

Autoradiography

After scintigraphy and roentgenography the forelegs from five randomly chosen animals were freeze-sectioned. Autoradiography was performed by apposition of the sections and remaining frozen block against Structurix D7 films (Agfa-Gevaert, Antwerpen, Belgium). Sections and films were kept under refrigeration during exposure. The technique for freeze-sectioning and autoradiography has been described by Ullberg (1954) and the application of the technique to this experimental design by Wittbjer, Palmer, Rohlin & Thorngren (1983). Some sections were stained with hematoxylin and eosin and then mounted.

RESULTS

In two rabbits the catheters were displaced during the experimental period, one died during anaesthesia and one was missing in the computer disc memory leaving 11 rabbits for evaluation.

Table II. Scintigraphic examination: Relative activity on side treated with hormone and side treated with saline of the radial defects at the centre and at sites of resection (proximal, distal)

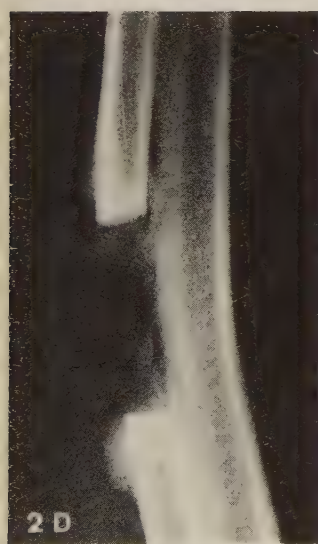
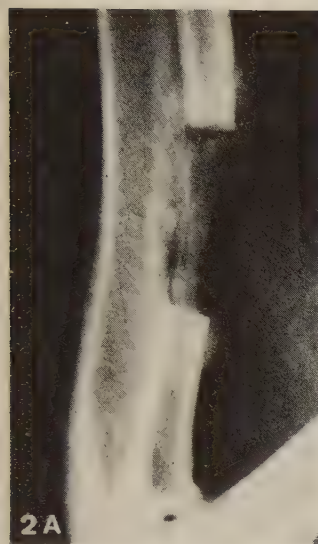
Rabbit no. xx	14 days						28 days					
	hormone			saline			hormone			saline		
	Proximal	Centre	Distal	Proximal	Centre	Distal	Proximal	Centre	Distal	Proximal	Centre	Distal
1	4.1	2.2	4.1	5.0	2.5	3.5	5.2	2.4	3.0	6.0	5.6	5.8
2	5.2	3.5	4.2	3.5	2.6	3.4	*	*	*	*	*	*
3	4.0	3.9	4.2	4.1	4.2	4.6	4.0	3.9	4.3	4.4	5.0	3.5
4	5.3	3.5	4.1	4.7	4.9	6.4	4.6	3.3	3.0	4.0	6.4	4.9
5	6.7	4.5	6.4	6.2	5.1	5.9	8.5	7.0	6.0	4.1	5.2	4.2
6	5.4	5.6	6.1	6.1	4.9	4.9	4.0	4.8	4.0	5.4	4.4	3.9
7	8.5	4.2	6.1	11.3	4.9	8.7	6.4	3.2	8.5	12.3	6.9	8.6
8	7.1	6.5	5.6	8.6	5.8	4.8	12.0	10.3	10.1	8.0	5.4	4.2
9	6.6	7.4	6.3	6.5	5.7	4.6	6.2	7.1	4.2	5.5	6.2	5.8
10	5.2	5.6	7.3	5.3	6.6	7.1	8.1	9.7	11.0	7.4	10.0	10.9
11	6.2	6.5	9.8	7.5	6.8	7.2	4.3	5.3	6.2	3.5	4.8	4.9
Mean	5.8	4.8	5.8	6.2	4.9	5.5	5.7	5.2	5.5	5.5	5.4	5.1

* Data lost in computer files

xx Rabbit no. corresponds to no. in Table I

Fig. 2. Roentgenographic and autoradiographic examinations (x 2) of left and right radius and ulna from an adult rabbit. Autoradiographic examination 4 hours after intravenous injection of $^{99}\text{Tc}^m$ -labelled DPD.

- A. Roentgenogram 14 days after operation of side treated with hormone. Mineralized tissue is seen in a large area of the defect. New mineral deposition is also present at the sites of resection on the outsides of radius.*
- B. Roentgenogram 28 days after operation of the side treated with hormone. The whole bone defect and also an area on the outside of the bone defect are filled with mineralized tissue.
The newly mineralized tissue has different opacity with two radiolucent areas (arrows).*
- C. Autoradiogram of the block reveals uptake of $^{99}\text{Tc}^m$ in a large area of the defect. No uptake is visible in two extended areas (arrows) corresponding to the radiolucent areas in Fig. 2 B.*
- D. Roentgenogram 14 days after operation of the side treated with saline. Newly mineralized tissue is seen in scattered areas inside the bone defect. The area of mineral deposition is smaller than that observed in Fig. 2 A.*
- E. Roentgenogram 28 days after operation of the side treated with saline showing mineral deposition in the whole bone defect. Similar to the pattern of mineral deposition seen in Fig. 2 B there are two extended areas with less mineral (arrows).*
- F. Autoradiogram of the block shows uptake of $^{99}\text{Tc}^m$ in three extended areas inside the bone defect. Two areas (arrows) inbetween without blackening correspond to the radiolucent areas in Fig. 2 E.*



Roentgenography

At the outside of the sites of resection a triangular radiopacity representing callus was seen in all rabbits (Figs 2A, 2D, 3A, 4A).

Fourteen days after operation the rabbits were examined alive, sometimes resulting in an image quality inferior to that obtained at examination of the specimens 28 days after operation. Radiopacity indicating mineralized tissue filled the bone defect to a varying degree (Table I) from minor (Fig. 2D) to extensive areas (Fig. 2A). There was no difference in the extent of mineralization between the sides.

Twenty-eight days after operation the areas of mineral deposition were occupying almost the whole bone defect (Table I). In six rabbits the extension of the mineralized tissue was equal (Figs 2B, 2E). In two rabbits there was a more extended mineral deposition on the side treated with hormone and in two rabbits on the side treated with saline. In many rabbits radiolucent lines were observed on both sides within the remineralized area. The radiolucencies corresponded possibly to the implanted matrix (Fig. 3). In most rabbits there was an extensive increase of the mineralized area between 14 and 28 days after operation representing a doubling of the amount of bone formed both at the side treated with hormone and at the side treated with saline.

Scintigraphy

Fourteen days after operation there was an even bone forming activity throughout the defect at both sides (Fig. 5). The activity in the centre of the defect at both sides was lower to that of the resection

ends, i.e. the callus formation (Table II). At twenty-eight days after operation no significant differences in activity were found between the hormone and saline treated sides (Fig. 6, Table II). Only a minor, not significant, increase in activity was noticed from 14 to 28 days at both sides when comparing the registered activity in the hormone and saline treated groups, respectively (Figs 5, 6, Table II).

Autoradiography

No difference in the degree or pattern of uptake of $^{99}\text{Tc}^{\text{m}}$ could be observed between the graft treated with hormone and the graft treated with saline. Most often the uptake was seen as three areas extending between the two sites of resection (Figs 2C, 2F, 3B, 4B, 4C).

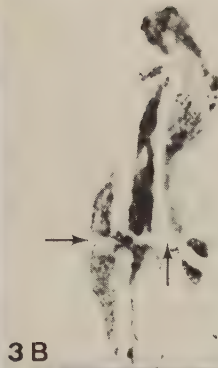
Inbetween these three areas there were two areas without radioactivity corresponding to remnants of implanted matrix (compare Figs 3B and 3C). In a few sections uptake of $^{99}\text{Tc}^{\text{m}}$ was observed in the whole defect (Fig. 4D). A triangular area of uptake was seen at the outlining of the radius corresponding to callus formation at the sites of resection (Figs 2C, 3B, 4B - 4D). Also at the periosteal and endosteal surfaces of radius and ulna an uptake of $^{99}\text{Tc}^{\text{m}}$ was seen. In thin areas between the sites of resection and the graft there was no uptake in some sections (Figs 3B, 4B, 4D).

Fig. 3. Radius and ulna in an adult rabbit 28 days after operation. This side has been treated with hormone.

- A. Roentgenogram (x 2) shows that almost the whole defect is filled with mineralized tissue. The deposition of mineral is heterogeneous with a somewhat striated pattern. Between the implant and end of resection there is a thin gap without mineral (arrows).*
- B. Autoradiogram (x 2) shows the uptake of $^{99}\text{Tc}^m$ 4 hours after intravenous injection of $^{99}\text{Tc}^m$ -labelled DPD. Uptake is seen in three areas with two areas without uptake inbetween. There is no uptake in a thin gap between the implant and radius (arrows).*
- C. Corresponding stained section (x 2) to Fig. 3 B. In two areas corresponding to the areas without uptake in Fig. 3 B, a homogeneous substance consisting of remnants of implanted bone matrix is seen.*

Fig. 4. Radius and ulna from an adult rabbit 28 days after operation. This side was treated with saline.

- A. Roentgenogram (x 2) reveals mineralized tissue in the whole bone defect. The mineral deposition is somewhat heterogeneous with radiolucent areas (arrows).*
- B,C,D. Autoradiograms (x 2) 4 hours after injection of $^{99}\text{Tc}^m$ -labelled DPD at different levels in the implant. All autoradiograms show uptake at the sites of resection as well as at the periosteal and endosteal surfaces of radius. In Fig. B no uptake is seen in the center of the implant and in areas close to the sites of resection (arrows). On a lower level (Fig. C) the uptake is more widespread centrally in the implant. On an even lower level (Fig. D) uptake is seen in almost the whole bone defect except for the area close to the sites of resection (arrow).*



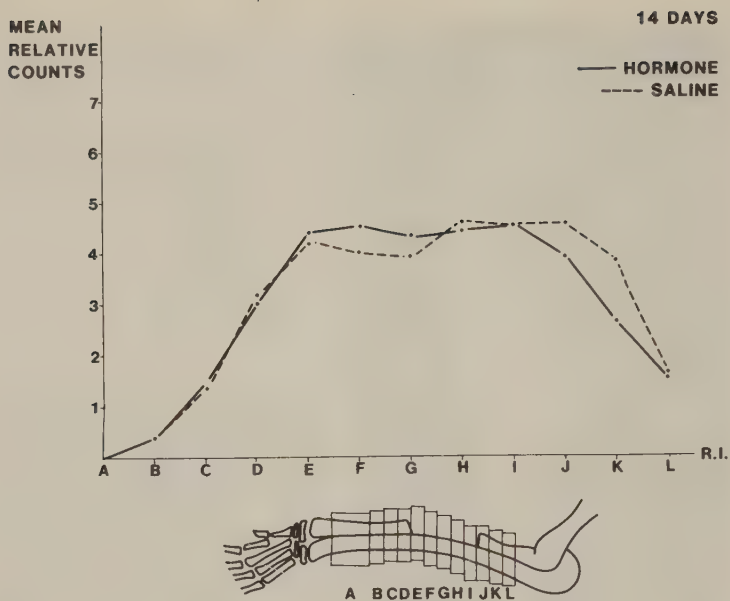


Fig. 5. Scintigraphic examination. Mean relative activity to the control area (A) in regions of interest (RI) 14 days after operation on the experimental side (hormone) and control side (saline). (Figures subtracted with 1 compared to the figures given in Table I).

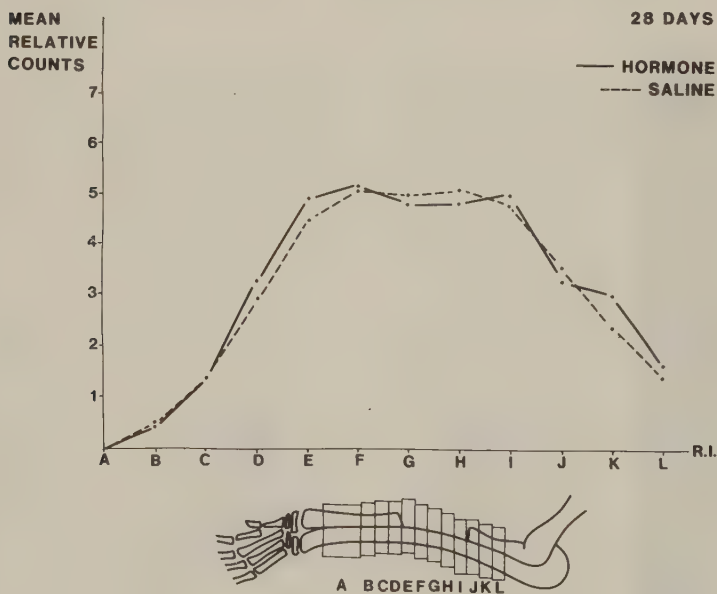


Fig. 6. Scintigraphic examination. Mean relative activity to the control area (A) in regions of interest (RI) 28 days after operation on the experimental side (hormone) and control side (saline). (Figures subtracted with 1 compared to the figures given in Table I).

DISCUSSION

Growth hormone given to adult dogs has resulted in an increase of bone formation and total skeletal volume (Mankin, Thrasher, Weinberg & Harris, 1978) and patients with acromegaly show bone trabeculae with increased thickness (Roelfsema, van der Sluys & Smeenk, 1970). In a patient with deficient amounts of growth hormone Misol, Samaan & Ponseti (1971) reported slow fracture healing. In conformity of this, positive results have been reported using growth hormone in treatment of slow healing fractures (Koskinen, Lindholm, Nieminen, Puranen & Attila, 1978; Ahl & Kalén, 1979). Simultaneous controls are, however, lacking in these studies.

Somatomedins have been proposed as intermediary substances for the growth promoting effects of growth hormone (Daughaday, Hall, Raben, Salmon, Van den Brande & Van Wyk, 1972; Giordano, Van Wyk & Minuto, 1979). This is based on the findings that growth hormone has only small or inconsistent effect on the metabolism of rat cartilage in vitro whereas serum from animals treated with growth hormone stimulates the tissue. Locally administered growth hormone in vivo would then have to be resorbed and induce somatomedin production, possibly in the liver (Skottner, Fryklund, Forsman & Castensson, 1980), enhancing the bone formation. Due to lacking in vivo effects in the hypophysectomized rat, however, this role of the somatomedins has been questioned (Kostyo & Isaksson, 1977; Isaksson, Jansson & Gause, 1982). In addition direct effects of growth hormone on the cartilage growth plate of hypophysectomized rats have recently been shown (Isaksson, Jansson & Gause, 1982), and Isaksson, Reagan & Kostyo (1976) suggested that only a brief encounter between growth hormone and its receptors on the outer surface of the plasma membrane is required to change the metabolic activity in the cells of target tissues. Thus, the initial event in action of growth hormone is its interaction with

tissue receptors, a process that generates a chain of reactions leading to accelerated protein synthesis and growth as suggested by Kostyo and Isaksson (1977).

Many species have a species specificity for growth hormone (Geschwind, 1966). Most mammals, the rabbit included, are, however, sensitive to human growth hormone, whereas a human being would not show any growth stimulation if given growth hormone from lower mammals (Geschwind, 1966). In hypophysectomized rats the biosynthetic growth hormone had in vivo effects on the bone formation similar to those of biologically produced growth hormone (Thorngren, Skottner-Lundin, Forsman, Löfberg, Fhølenhag & Fryklund, 1983).

The recommended clinical systemic dose for HGH would converted correspond to 0.3 IU per rabbit per day. A subcutaneous dose of 0.5 mg bovine growth hormone daily for 10 days (corresponding 5 IU) to adult rabbits resulted in mitosis of chondrocytes in the knee joints (Henriksson, Havdrup & Telhag, 1982). The daily dose used in the present investigation was 0.17 IU for 14 days corresponding to a total dose of 2.4 IU. As it was concentrated by local deposition to the tissue inside the bone defect the dose ought to be high enough for potential influence on bone formation.

Usually growth hormone is administered intermittently as one daily injection, but more frequent injections of a total equivalent dose increase the growth response (Thorngren & Hansson, 1977; Jansson, Albertsson-Wikland, Edén, Thorngren & Isaksson, 1982a), and circumstantial evidence has been presented for a role of the secretory pattern of growth hormone in control of body growth

(Jansson, Albertsson-Wikland, Edén, Thorngren & Isaksson, 1982b).

Continuous administration of growth hormone, however, also resulted in growth promotion (Cotes, Barlett, Gaines Das, Flecknell & Termeer, 1980) and the continuous excessive dose used in the present investigation would therefore be effective. A continuous infusion would guarantee continuous saturation of available receptors for growth hormone on the outer surface of the plasma membrane (Isaksson, Reagan & Kostyo, 1976). Thus, it seemed advantageous to use the osmotic pump tested in previous investigations by Cotes, Barlett, Gaines Das, Flecknell & Termeer (1980) and guaranteed by the manufacturer for delivering a solution continuously for 14 days. Besides, daily injections into the radial bone defect were considered more laborious to the rabbit than the closed circuit.

No difference in bone forming activity was noticed between the hormone and saline treated sides neither 14 nor 28 days after operation. In the roentgenograms of both sides independent of hormone treatment there were central radiolucent areas in the remineralized bone transplant making the roentgenographic evaluation difficult. Due to this the remineralization after 28 days was probably overestimated in comparison to a study with a similar design (Wittbjer, Palmer, Rohlin & Thorngren, 1983), whereas the roentgenographic evaluation of the two sides in the present study was systematically subjected to this error. Such a pattern for the deposition of mineral has not been observed in previous study of the remineralization of matrix in bone defects (Wittbjer, Nosslin, Palmer & Thorngren, 1982; Wittbjer, Rohlin & Thorngren, 1983 ; Wittbjer, Glantz, Rohlin & Thorngren, 1983).By the aid of autoradiography and corresponding stained sections this pattern

caused by large remnants of matrix was more conspicuous. The slow degradation of matrix might be a consequence of the flow of solutions in the region of both sides resulting in a mechanical wash-out effect and/or a dilution of biochemical components (enzymes) and reduction of cellular elements pertinent to the degradation of matrix.

The fact that the bone forming activity was about the same on the hormone and saline treated sides might also be due to that the growth hormone during the first 14 days neither influenced the degradation of the implanted collagen matrix to bone morphogenetic protein (Urist, Mikulski & Lietze, 1979) nor the primary induction transforming histiocytes to bone precursor cells (Urist, 1970). In a previous study (Wittbjør, Palmer, Rohlin & Thorngren, 1983) it was found that adding fresh marrow cells to implanted matrix accelerated the bone formation during the first 14 days. Between 14 and 28 days the bone formation of demineralized matrix levelled the osteoblastic activity of the composite graft of demineralized matrix with marrow. This indicates that there is an increasing amount of osteoblasts through the experimental period not reaching its peak until 14 days after operation. As shown by Handelsman & Gordon, (1930); Frost, (1962); Harris, Heaney, Jowsey, Cockin, Akins, Graham & Weinberg, (1972), growth hormone has an accelerating effect on the osteoblastic activity. No effects on the healing of fresh fractures were, however, found neither experimentally (Harris, Bean & Banks, 1975; Northmore-Ball, Wood & Meggitt, 1980) nor clinically (Lindholm, Koskinen, Puranen, Nieminen, Kairaluoma & Attila, 1977), whereas positive results were reported using growth hormone in treatment later in the healing process of fractures (Koskinen, Lindholm, Nieminen, Puranen & Attila, 1978; Ahl & Kalén, 1979). Bearing this in mind there would probably have

been a more pronounced difference in bone forming activity between the hormone and saline treated sides in the present study if the hormone treatment had lasted throughout the experimental period or started at 14 days after operation. At the time for this investigation no osmotic pumps delivering solutions for more than 14 days were, however, available. Also, it seemed as growth hormone had a positive effect only if the bone defect was large enough to result in deficient healing in the normal bone formation process (Zadek & Robinson, 1961).

In view of the present finding it might be of interest to study both the effect of prolonged growth hormone treatment as well as systemic growth hormone treatment and the addition of bone forming cells. As the addition of fresh marrow cells to a demineralized implant increased the bone forming activity during the first 14 days after operation (Nade & Burwell, 1977; Wittbjer, Palmer, Rohlin & Thorngren, 1983) the influence of a combination of fresh marrow cells and growth hormone on the bone formation in demineralized bone matrix implants might be of interest to study.

REFERENCES

- Ahl, T. & Kalén, R. 1979. Effect of human growth hormone in the treatment of pseudoarthrosis. *Opuscula Medica* 1, 26.
- Cotes, P.M., Barlett, W.A., Gaines Das, Flecknell, P. & Termeer, R. 1980. Dose regimens of human growth hormone: Effects of continuous infusion and of a gelatin vehicle on growth in rats and rate of absorption in rabbits. *J Endocrinol* 87, 303.
- Daughaday, W.H., Hall, K., Raben, M.S., Salmon, W.D.Jr., Van Den Brande, J.L. & Van Wyk, J.J. 1972. Somatomedin: Proposed designation for sulfation factor. *Nature* 236, 107.
- Edén, S., Isaksson, O.P.G., Madsen, K. & Friberg, W. 1983. Specific binding of growth hormone to isolated chondrocytes from rabbit ear and epiphyseal plate. *Endocrinology*. (In press).
- Friedlaender, G.E. 1982. Current concepts review bone-banking. *J Bone Joint Surg* 64-A, 307.
- Frost, H.M. 1962. A model of endocrine control of bone remodelling. *Henry Ford Hosp M Bull* 10, pt. 2, 119.
- Geschwind, I.I. 1966. Species specificity of anterior pituitary hormones. In *The Pituitary Gland* (eds. G.W. Harris & B.T. Donovan), vol. 2, pp. 589-612. Butterworths, London.
- Giordano, G., Van Wyk, J.J. & Minuto, F. (eds.). 1979. Somatomedins and Growth. *Proceedings of the Sero Symposia*, vol. 23, p. 363. Academic Press, London.
- Handelsman, M.B. & Gordon, E.F. 1930. Growth and bone changes in rats injected with anterior pituitary extract. *J Pharmacol Exp Ther* 38, 349.

- Harris, J.A., Bean, D.A. & Banks, H.H. 1975. Effect of phosphate supplementation, thyrocalcitonin and growth hormone on strength of fracture healing. *Surgical Forum* 26, 519.
- Harris, W.H., Heaney, R.P., Jowsey, J., Cockin, J., Akins, C., Graham, J. & Weinberg, E.H. 1972. Growth hormone: The effect on skeletal renewal in adult dog. *Calcif Tissue Res* 10, 1.
- Henriksson, A.S., Havdrup, T. & Telhag, H. 1982. The effect of growth hormone and thyroxine on adult joint cartilage. *Clin Orthop* 162, 270.
- Isaksson, O.G.P., Jansson, J.-O. & Gause, I.A.M. 1982. Growth hormone stimulates longitudinal bone growth directly. *Science* 216, 1237.
- Isaksson, O., Reagan C. & Kostyo, J. 1976. Use of antibodies to modify the interaction of growth hormone (GH) with isolated muscle. *Fed Proc* 35, 783.
- Jansson, J.-O., Albertsson-Wikland, K., Edén, S., Thorngren, K.-G. & Isaksson, O. 1982a. Effect of frequency of growth hormone administration on longitudinal bone growth and body weight in hypophysectomized rats. *Acta Physiol Scand* 114, 261.
- Jansson, J.-O., Albertsson-Wikland, K., Edén, S., Thorngren, K.-G. & Isaksson, O. 1982b. Circumstantial evidence for a role of the secretory pattern of growth hormone in control of body growth. *Acta Endocrinol (Copenh)* 99, 24.
- Koskinen, E.V.S., Lindholm, R.V., Nieminen, R.A., Puranen, J.P. & Attila, W. 1978. Human growth hormone in delayed union and non-union of fractures. *Int Orthop* 1, 317.
- Kostyo, J.L. & Isaksson, O. 1977. Growth hormone and the regulation of somatic growth. In *International review of physiology* (ed. R.O. Greep). *Reproductive Physiology II*, vol. 13, pp. 255-274. Baltimore.
- Lindholm, R.V., Koskinen, E.V.S., Puranen, J., Nieminen, R.A., Kairaluoma, M. & Attila, W. 1977. Human growth hormone in the treatment of fresh fractures. *Horm Metab Res* 9, 245.

- Mankin, H.J., Thrasher, A.Z., Weinberg, E.H. & Harris, W.H. 1978. Dissociation between the effect of bovine growth hormone in articular cartilage and in bone of the adult dog. *J Bone Joint Surg* 60-A, 1071.
- Misol, S., Samaan, N. & Ponseti, I.V. 1971. Growth hormone in delayed fracture union. *Clin Orthop* 74, 206.
- Nade, S. & Burwell, R. 1977. Decalcified bone as a substrate for osteogenesis. An appraisal of the interrelation of bone and marrow in combined grafts. *J Bone Joint Surg* 59B, 189.
- Northmore-Ball, M.D., Wood, M.R. & Meggitt, B.F. 1980. A biomechanical study of the effects of growth hormone in experimental fracture healing. *J Bone Joint Surg* 62-B, 391.
- Reddi, A.H. & Sullivan, N.S. 1980. Matrix induced endochondral bone differentiation: Influence of hypophysectomy, growth hormone, and thyroid stimulating hormone. *Endocrinology* 107, 1291.
- Roelfsema, F., van der Sluys, J. & Smeenk, D. 1970. Quantitation of bone and bone turnover in biopsy specimens from the iliac crest in acromegaly. *J Endocrinol* 48, 61.
- Skottner, A., Fryklund, L., Forsman, A. & Castensson, S. 1980. Somatomedins A and B, Biological studies. In *Growth hormone and other biological active peptides* (eds. Pecile, A. & Müller, E.E.). *Excerpta Medica International Congress Series*, Amsterdam, 495, pp 65-72.
- Stenström, A., Hansson, L.-I. & Thorngren, K.-G. 1976. Effect of growth hormone on cortical bone remodelling in hypophysectomized rat. In *Hormonal influence on cortical bone remodelling* (ed. Stenström, A). Thesis. Studentlitteratur, Lund.
- Thorngren, K.-G. 1973. Growth hormone and longitudinal bone growth. *Comm Dept Anat (Lund)* 9, 1.

- Thorngren, K.-G. & Hansson, L.I. 1977. Effect of administration frequency of growth hormone on longitudinal bone growth in the hypophysectomized rat. *Acta Endocrinol (Copenh)* 84, 497.
- Thorngren, K.-G., Skottner-Lundin, A., Forsman, A., Lövberg, E., Fhølenhag, K. & Fryklund, L. 1983. Bone growth stimulation by biosynthetic methionyl human growth hormone. Submitted to *Acta Orthop Scand*
- Ullberg, S. 1954. Studies on the distribution and fate of S^{35} -labelled benzyl-penicillin in the body. *Acta Radiol (Suppl)* 116.
- Urist, M.R. 1970. The substratum for bone morphogenesis. *Dev Biol (Suppl)* 4, 125.
- Urist, M.R., Mikulski, A.J. & Lietze, A. 1979. Solubilized and insolubilized bone morphogenetic protein. *Proc Natl Acad Sci* 76, 1828.
- Wittbjer, J., Glantz, P.-O., Rohlin, M. & Thorngren, K.-G. 1983. On direct current and bone formation in demineralized bone transplants. Submitted to *Acta Odontol Scand*
- Wittbjer, J., Nosslin, B., Palmer, B., & Thorngren, K.-G. 1982. Bone formation of transplanted autologous bone matrix in rabbit evaluated by technetium radionuclide bone imaging. *Scand J Plast Reconstr Surg* 16, 23.
- Wittbjer, J., Palmer, B., Rohlin, M. & Thorngren, K.-G. 1983. Osteogenetic activity in composite grafts of demineralized compact bone and marrow. *Clin Orthop* 173, 255.
- Wittbjer, J., Palmer, B. & Thorngren, K.-G. 1982. Osteogenetic properties of reimplanted decalcified and undecalcified autologous bone in the rabbit radius. *Scand J Plast Reconstr Surg* 16, 239.
- Zadek, R.E. & Robinson, R.A. 1961. The effect of growth hormone on healing of an experimental long-bone defect. *J Bone Joint Surg* 43-A, 1261.

ON DIRECT CURRENTS AND BONE FORMATION IN DEMINERALIZED
BONE TRANSPLANTS

J. Wittbjer, P.-O. Glantz, M. Rohlin & K.-G. Thorngren

From the Maxillofacial Centre, Departments of Plastic
Surgery and Nuclear Medicine, Malmö General Hospital
and Departments of Prosthetic Dentistry, Oral Radiology
and Orthopaedic Surgery, University of Lund, Sweden

Address: Dr. Jan Wittbjer, Maxillofacial Centre,
Malmö General Hospital, S-214 21 Malmö,
Sweden

ABSTRACT

The effect of direct current on the remineralization of a bone defect was studied in rabbit. Bone defects in the radius of both fore-legs were grafted with demineralized autologous bone. On both sides platinum electrodes were encircled around the graft and one side was connected to a power source delivering 20 μ A constant current during the experimental period of 28 days. The remineralization was evaluated at 14 and 28 days after operation by scintigraphy and roentgenography, planimetry included. At 28 days after operation this evaluation was supplemented by autoradiography.

Roentgenographically there was no difference between the two sides. At 14 days after operation scintigraphy demonstrated a minor delay in bone formation at the electrostimulated side. Between 14 and 28 days a significant increase in activity was noticed. On both sides, autoradiograms revealed areas without uptake around the wires. It was concluded that direct currents of the studied magnitude have a negative influence on the primary bone induction process but also that it seems to influence the mineralization positively later in the bone forming process.

INTRODUCTION

The first attempts to stimulate bone healing by electricity date to the middle of the 19th century (1). The hypothesis that electrically generated energy could stimulate the growth- and healing processes in bone tissue evoked particular interest when Yasuda (35) published a now classical paper demonstrating that bone had stressgenerated piezoelectric properties and that direct current of one μA over a three week period could produce callus around a cathode placed in bone. Corresponding results of in vivo and in vitro studies have been reported for example by Basset (1), Basset and Becker (4), Shamos and Lavine (25), Basset (2), Herbst (14) and ElMessiery (11). A large number of clinical studies have reported success with this method in up to 80 per cent of cases of nonunion and pseudoarthrosis (16, 18, 7, 3).

Basicly, two different types of systems are presently used clinically. Stimulation is either carried out by direct currents in an invasive form by implantable stimulators (18, 7) or by pulsing electromagnetic fields in a noninvasive system (3). Basset (3) claimed that pulsing electromagnetic fields have an implication on clinical situations that far exceeds that of constant direct currents. The main reason is that pulsing systems are noninvasive and that they do not give rise to the anodic toxic effects often observed in experiments with direct currents. To minimize the negative effects around the anodes of implantable devices, pulsing current has been introduced (20, 24). According to Dwyer and Wickham (10) as well as Paterson, Lewis and Cass (18) there

is no definite difference in the osteogenic capacity between direct and pulsing current methods.

Autologous demineralized bone in a radial bone defect has previously been shown to have better osteogenic properties than autologous bone containing mineral (34).

Since electricity has clinically been claimed to accelerate bone formation in bone defects it was considered of interest to study the effect of electric stimulation on the mineralization process in a bone defect grafted with autologous demineralized bone. As rabbit was used as experimental animal it was decided to use an implantable system for electrostimulation generating direct currents.

MATERIALS and METHODS

General outline:

Nineteen adult rabbits with an average body weight of 3.5 kg were used. The operation was performed under anaesthesia consisting of intramuscular injection of Hypnorm Vet[®] (Leo, Helsingborg, Sweden) and intravenous injection of Mebumal (ACO, Solna, Sweden) supplemented with local anaesthesia (Xylocain[®] 0.5 %, Astra, Södertälje, Sweden).

Initially a 12 mm piece of the radius was resected bilaterally and demineralized in 0.6 N HCl for 24 hours. After a thorough rinsing in saline these pieces were kept at +4° C for two days and in a second operation reimplanted in the defects with triple helix electrodes (cathodes) surrounding the demineralized pieces of bone. The materials and methods used at these operations are described in details by Wittbjer,

Nosslin, Palmer and Thorngren (32). The implanted electric stimulators generated a constant current of 20 μ A during the experimental period of 28 days.

At 14 days after operation the remineralization process was evaluated by roentgenography and scintigraphy. At 28 days the rabbits were killed and the same evaluation was performed supplemented with autoradiography (33).

Electrostimulator: The implanted electrostimulator was equipped with six BEREC[®] PX/RM 400 1.35 V Mercury cells. It had a field effect transistor in series with a resistor controlling the circuit delivering 20 μ A constant current (19). Two platinum-electrodes with a length of 40 mm and a diameter of 0.5 mm were connected to the stimulator through polyeten insulated stainless steel wires.

The electrostimulating device was surgically implanted into the back of the rabbit and its electrodes tunneled subcutaneously to the radial defect. The anode was placed over the muscle fascia on the medial side of the humerus at a distance of approx. 40 mm from the defect area. The helical cathode with the inserted bone matrix was placed in the radial defect and fixed with sutures. The wounds were closed in anatomical planes. The procedures described were performed randomly either on the right or left fore-legs of the rabbits. The platinum-wire and bone matrix were placed in the same manner on the experimental as on the contralateral control side, but without connection to a stimulator. An outline of the

experimental arrangements is given in Fig. 1. After sacrifice the stimulators were tested to verify that constant current levels were generated.

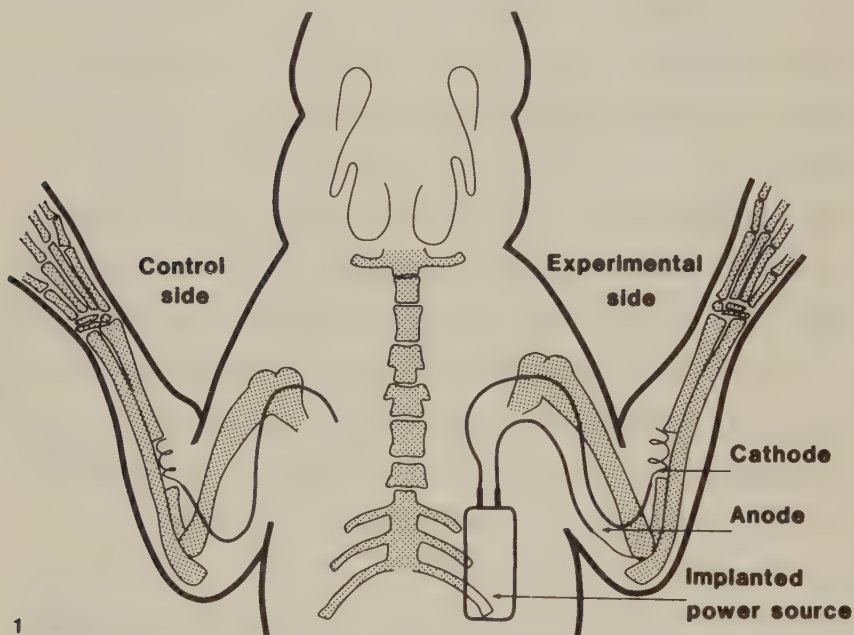


Fig. 1. Implanted power source with electrodes leading to the radial defects. On one side the electrode is connected to the power source

Roentgenography: The bones were examined with a dental X-ray unit (Oralix 65, N.V. Philips, Eindhoven, Netherlands) using Kodak Occlusal Ultra-Speed D films and an exposure time of 0.4 second. During the roentgenographic and scintigraphic examination 14 days after operation the rabbit was anaesthetized with Hypnorm Vet[®]. Twenty-eight days after the operation the right and left fore-leg

specimens were placed on the same X-ray film. The resulting roentgenograms were placed in an X-ray film enlarger/viewer (Realist Inc. Photographic Products Division, Wisconsin, U.S.A.) with an optic magnification of 10:1. The display was covered with transparent paper on which the outlines of the bone defect and the remineralized tissue inside the bone defect were drawn. The remineralized area was then estimated with a planimeter (Los Angeles Scientific Instruments Co., Los Angeles, U.S.A.). The extent of the remineralized area was expressed in percentage of the area of the initial bone defect. The extent of mineralization was judged to be equal on the two sides when the difference was three per cent or less.

Scintigraphy: $^{99}\text{Tc}^{\text{m}}$ -DPD, a technetium -99m labelled 3.3 diphosphono- 1.2 -propanedicarboxylic acid, tetra sodium salt (Behringwerke AG, Marburg, West Germany) was injected intravenously three hours before the animals were killed. The injected solution contained an activity of approx. 350 MBq. Using the methods described by Wittbjør et al. (32) the gamma radiation was registered and mean counts were calculated in regions mapped out covering the defect area. Relative values to a control area outside the defect were then calculated. The mean values in every region (Figs. 5, 6) and the relative values in the center, proximal and distal part of the defect at the experimental side (active cathode) and at the control side (passive cathode) were also calculated (Table 2, Fig. 7).

Autoradiography: After scintigraphy and roentgenography and four hours after injection of $^{99}\text{Tc}^{\text{m}}$ -DPD the fore-leg specimens from seven randomly chosen animals were freeze sectioned. Autoradiography was then performed by apposition of the sections and remaining frozen block against Structurix D7 films (Agfa-Gevaert, Antwerpen, Belgium). Sections and films were kept under refrigeration during exposure and then developed and fixed. The technique for freeze sectioning and autoradiography has been described in detail by Ullberg (30) and the autoradiographic technique for $^{99}\text{Tc}^{\text{m}}$ -labelled sections by Rohlin and Hammarström (23). The modifications of this technique to the experimental design used in this study have been described by Wittbjer et al. (33). Selected sections were stained with hematoxylin and eosin and then mounted.

RESULTS

In 8 rabbits the electrodes were disconnected from the power cells or displaced from the experimental areas during the experimental period leaving 11 rabbits for evaluation.

Roentgenography: At the outsides of the radii at the sites of resection a triangular radiopacity representing callus was seen (Figs. 2, 3A, 4A).

Fourteen days after the operation the rabbits were examined alive and therefore the image quality sometimes was not quite satisfactory due to movements during the examination. In average there was no significant difference in extent of remineralization between the side treated with active electrode (Table 1). More extended mineralization on the side treated with active electrode was observed in four rabbits whereas in three rabbits less mineralized tissue was observed. The extent of the mineralized tissue was judged to be equal (compare Figs. 2A and 2B) in four rabbits.

Twenty-eight days after operation the extent of mineralization was somewhat less on the side treated with active electrode compared to passive electrode side (Table 1). This difference was particularly pronounced in one rabbit (No. 6). With this rabbit excluded the mean values for mineralized tissue would be equal for the two sides. In three rabbits more extended mineral deposition was seen on the side treated with active electrode (compare Figs. 2C and 2D) and on the side treated with passive electrode

Table 1. Roentgenographic examination 14 and 28 days after operation: Planimetric evaluation of mineralized areas in percentage of the radial initial bone defect

Rabbit no.	14 days		28 days	
	active electrode	passive electrode	active electrode	passive electrode
1	19	20	66	94
2	57	62	99	99
3	21	5	75	73
4	39	40	87	57
5	31	35	43	58
6	20	29	23	98
7	28	30	100	89
8	70	31	100	100
9	94	91	100	100
10	26	24	51	35
11	45	32	85	98
Mean	41	36	75	82

Table 2. Scintigraphic examination 14 and 28 days after operation: Relative activity of the radial defects at the center and at the sites of resection (proximal, distal)

Rabbit no.†	14 days				28 days						
	Active electrode		Passive electrode		Active electrode		Passive electrode				
	Proximal	Center	Distal	Proximal	Center	Distal	Proximal	Center	Distal		
1	5.5	2.9	4.9	*	*	8.4	6.1	5.3	4.1	3.6	6.4
2	3.5	2.3	4.1	6.4	5.7	7.4	13.0	5.9	8.1	10.9	7.9
3	7.6	5.9	8.2	6.1	3.8	6.0	8.2	7.7	7.8	5.2	4.9
4	5.7	5.2	7.5	8.3	5.8	8.7	5.1	5.6	4.7	5.3	6.0
5	4.1	2.0	4.1	5.2	3.3	6.5	3.5	2.1	3.5	2.0	4.8
6	4.5	2.6	4.0	8.0	3.7	5.9	5.7	3.5	5.9	7.4	7.5
7	7.9	6.9	8.4	9.8	9.9	10.8	4.8	6.7	5.5	5.8	5.6
8	6.0	7.3	6.1	4.5	5.1	3.4	5.7	7.6	6.4	*	*
9	4.8	5.0	4.3	5.6	6.5	3.8	7.7	8.6	6.7	5.5	6.0
10	*	*	*	3.9	4.4	3.8	4.2	3.4	4.2	4.3	4.8
11	3.9	3.6	5.0	4.8	5.6	4.3	*	*	*	*	*
Mean	5.4		5.7	6.3	5.4	6.1	6.2		5.6	5.6	6.0
	4.4				p < 0.02 **				6.4		

* Data lost in computer files

** p-value according to Wilcoxon's matched pairs signed-ranks test

† Rabbit no. corresponds to no. in Table 1

Fig. 2. Roentgenograms of right and left radius and ulna from adult rabbit 14 days (A, B) and 28 days (C, D) after operation. Left side with active electrode and right side with passive electrode ($\times 2$).

Fig. 2 A, B. There are scattered areas of mineralized tissue in the bone defect. The extent of mineralized tissue is about the same on the two sides.

Fig. 2 C, D. The extent of mineralized tissue has increased considerably compared to Figs. A, B. On the side with active electrode there is somewhat more mineralized tissue. On both sides areas without mineral are seen. At the sites of resection there is callus formation on the outside of radius. In the defect area periosteal thickening is seen on ulna.



Fig. 3. Roentgenograms (A) and autoradiograms at three sagittal levels (B, C, D) of radius and ulna from adult rabbit 28 days after operation. The inserted electrode was active (x 2).

Fig. 3 A. Almost all tissue inside the bone defect is mineralized. There is, however, small radiolucent areas inside the newly mineralized tissue (arrows).

Fig. 3 B, C, D. Uptake of $^{99}\text{Tc}^m$ 4 hours after intravenous injection of $^{99}\text{Tc}^m$ -labelled DPD. The extent of uptake varies with level of sectioning. Centrally an area without uptake is observed. Compared to Fig. 3 A the extent of mineral deposition is smaller in the three sections.

Fig. 4. Radius and ulna in an adult rabbit 28 days after operation. The wire was not connected to electrostimulator (x 2).

Fig. 4 A. Roentgenograms show that almost the whole bone defect is filled with mineralized tissue. Close to the wire there are radiolucent areas.

Fig. 4 B. Autoradiograms reveal the uptake of $^{99}\text{Tc}^m$ 4 hours after intravenous injection of $^{99}\text{Tc}^m$ -labelled DPD: The uptake is seen in a large area of the defect. Well-defined areas without uptake lay centrally (arrows).

Fig. 4 C. Corresponding sections to Fig. 4 B. Around the wires (arrows) well-defined areas without tissue are seen.



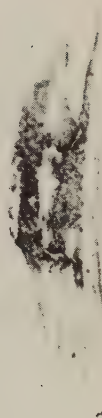
3 B



3 C



3 D



4 B



4 C



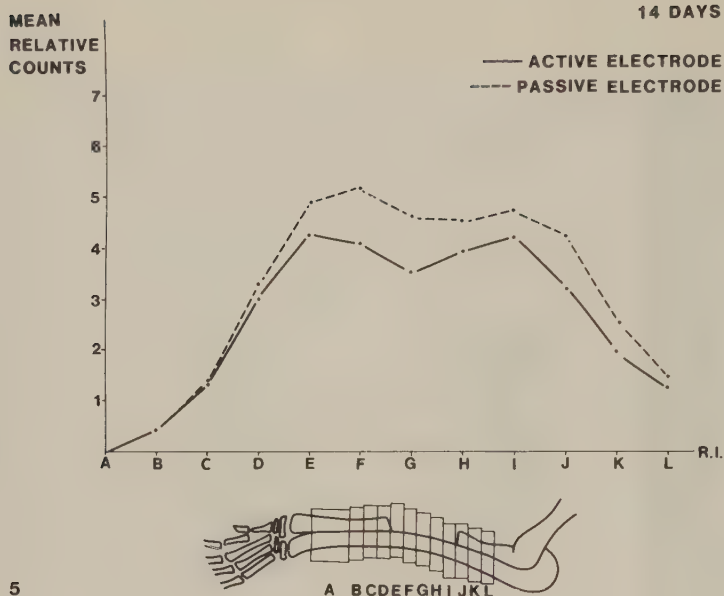


Fig. 5. Scintigraphic examination. Mean relative activity to the control area (A) in regions of interest (RI) 14 days after operation on the experimental side (active electrode) and control side (passive electrode). (Figures subtracted with 1 compared to the figures given in table 2).

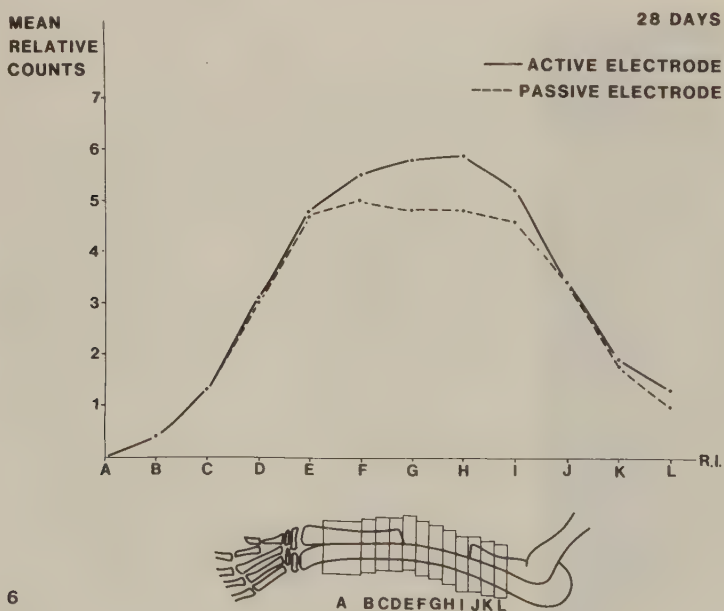


Fig. 6. Scintigraphic examination. Mean relative activity to the control area (A) in regions of interest (RI) 28 days after operation on the experimental side (active electrode) and control side (passive electrode). (Figures subtracted with 1 compared to the figures given in table 2).

in four rabbits. In remaining four rabbits the extent of mineralized tissue was judged to be equal on the two sides.

Scintigraphy: As observed in Fig. 5 a slightly decreased osteogenic activity could be observed after 14 days on the experimental side as compared with the control. No statistically significant differences (Wilcoxon matched pairs signed-rank test) were, however, observed between the values for the two sides.

After 28 days, as is given in Fig. 6, the activity was found to be somewhat higher on the experimental side. Neither were here any statistically significant differences found between the results of the two sides.

When the values obtained after 14 and 28 days were compared (Fig. 7, Table 2) a definite increase in bone formation activity was found to be present on the experimental side. This difference was found to be statistically significant ($p < 0.02$) in the center of the defect.

Autoradiography: Uptake of $^{99}\text{Tc}^{\text{m}}$ ranged from extensive (Figs. 3C, 4D) to small areas of the bone defect (Figs. 3B, 3D, 4B). The extent of uptake varied with level of sectioning in the same specimen (Figs. 3B, 3C, 3D) at some levels similar to the areas with newly mineralized tissue revealed by roentgenography (compare Figs. 3A and 3C). However, the areas with uptake were most often smaller than the areas with mineralized tissue in corresponding roentgenograms. Several autoradiograms and corresponding stained sections

revealed small well-defined areas without uptake or tissue (Figs. 3D, 4B, 4C) often located around the electrodes irrespective of active or passive electrode (Figs. 3D, 4B). No remnants of unresorbed bone matrix could be seen in the stained sections. Small thin areas without uptake were located between the implant and the sites of resection in some sections (Fig. 4D).

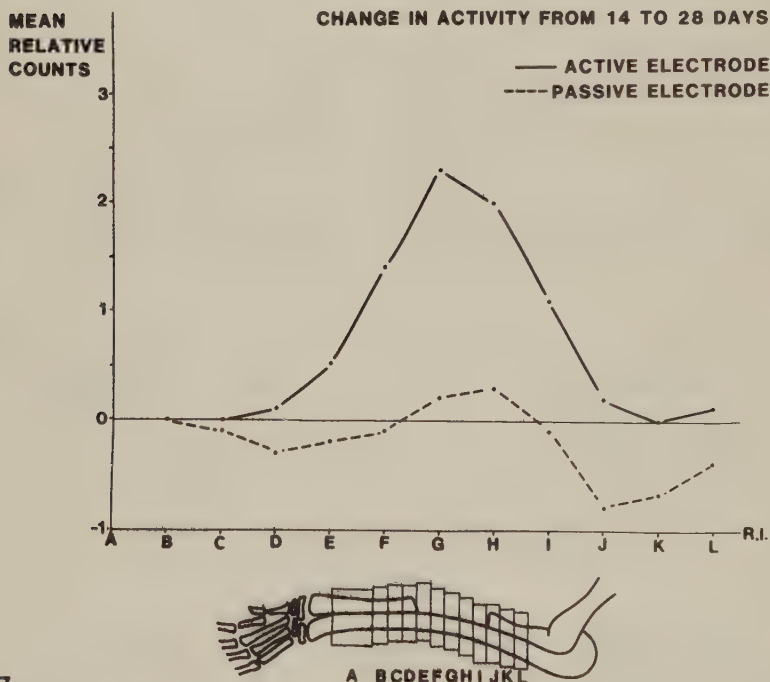


Fig. 7 Scintigraphic examination. The difference in mean relative activity to the control area (A) in regions of interest (RI) from 14 and 28 days after operation on the experimental side (active electrode).

DISCUSSION

There is a lack of detailed knowledge about the biological processes behind the electrostimulating enhancement of bone formation. According to Davidovich and Shanfeld (9) the stimulus provided by bioelectrical currents may involve an initial depolarization of cell membranes. Metabolically based differences in electrical potential frequently exist across layers of cells (29) and since ionic transport across bone membranes results in differences in electrical potential such differences could perhaps promote osteogenesis. Bearing in mind the relatively low current densities used in this study it is, however, reasonable to assume that the currents had comparatively minor influence on the transport of ions in the examined tissues. Considering this Justus and Luft (17) suggested a mechanochemical hypothesis regulating extracellular calcium influencing the osteoblastic activity. Such events could not be evaluated by methods of the present study which reveals presence of mineral. However, for clinical applications it is important to evaluate the quality and quantity of bone tissue formed.

It was considered important to select for the cathode a metal or metal alloy having electrochemical properties with minimal toxic reactions. Platinum was selected as it has been shown to be a suitable electrode material and more osteogenic than for example titanium and stainless steel (26). Probably this is because of its favourable electrochemical properties and ability to catalyze the oxygen reactions believed to be of importance in bone formation (6).

Friedenberg et al. (13) examined five different electrode positions in relation to fracture location. Best results were obtained with the cathode in the fracture and the anode in bone tissue or soft tissue at some distance from the fracture. Similar arrangement was used in the present study.

In clinical studies as well as in experimental ones varying amounts of direct current have been used. At current levels above 10 μA anodic necrosis has been observed and at levels above 100 μA also necrosis around the cathode was observed (12). As an adequate current level for osteogenesis was considered to be within the range of 5 - 20 μA , with an optimum effect at 20 μA (12, 27, 15), this current level corresponding to an approximate current density of $0.3 \mu\text{A}/\text{mm}^2$ was used in the present experiments. Furthermore, the current level of 20 μA is also used for clinical treatment of pseudoarthroses (7, 19).

After two weeks the scintigraphic evaluation revealed a slight negative osteogeneic effect from electric currents in this study. However, after four weeks the influence evaluated by scintigraphy had turned into a slight positive one confirming results presented by Petersson, Holmer and Johnell (19). The differences between the two observation periods were found to be statistically significant for the side with electric current. According to Rodan et al. (21, 22) small electrical currents provide a "trigger stimulus" or threshold that initiates a sequence of cellular events. These involve the proliferation of stem cells and their subsequent differentiation into an osteogeneic cell line.

At the beginning of the observation period in the present study matrix but few cells were available for stimulation. According to Spadaro (26) and Basset (3) it is conceivable that optimal field strength differs at each stage of healing since different cells are involved at different times. It is most likely that the electric current disturbed that initial inductive influence from the implanted matrix which the implant previously was shown to have in this experimental design (32). Compared to these findings less mineralization and bone forming activity to some extent confirms the statement. Later in the bone forming process when an adequate amount of osteoblasts are present there is a significant increase in bone forming activity compared to 14 days after operation. These findings can be due to the earlier noticed delay in the osteogenesis but also to a possible appropriate field strength influencing the metabolic activity of the cells. Such a reaction is supported by the findings of Herbst, Lindskog and Hammarström (15) that electrostimulated bone formation in tissue cultures was associated with an increase in the metabolic activity rather than an increase in the quantity of cells.

In pseudoarthroses the osteogeneic activity of electricity is believed to be associated either with a stimulation of the collagen matrix to act as a receiver for hydroxyapatite (8) or with an alteration of the proteoglycanrich cartilage creating conditions for mineralization (5). However, the collagen structure in the fibrous or fibrocartilaginous tissues bridging a pseudoarthroses has no inductive capacity of its own (31) and thereby differs from the matrix

of implants used in the present study.

In this study it was found well-defined areas around both wires without uptake revealed in autoradiograms and lack of tissue close to the wires seen in the stained sections. This could be interpreted as reaction towards the metal and is in contrast to the findings of Spadaro (26) demonstrating platinum to stimulate osteogenesis even without current. As this appeared on both sides independently of electrical stimulation the level of current could not have caused the reaction. A continuous movement of the wires, however, might have obstructed the aggregation of cells resulting in the well-defined borderlines of the areas.

Summing up, the observed lack of definite electrostimulating effects on bone formation in demineralized bone do not indicate that electricity per se has no influence on osteogenesis. It rather indicates that electrical stimulation might be more effective in a later phase of bone formation similar to the effect described in pseudoarthroses and fibrous tissue bridging a non-healed bone defect or effective in stimulation of existing bone forming cells.

ACKNOWLEDGEMENT

Supported by the Faculties of Odontology and Medicine, University of Lund, by Stiftelsen Konsul Thure Carlssons Minne and by the Swedish Medical Research Council (Project No. 17X-2031).

REFERENCES

1. Basset, C.A.L. Current concepts of bone formation
J. Bone Joint Surg. 1962, 44-A, 1217-1244
2. Basset, C.A.L. Biophysical principles affecting bone structure. In: The biochemistry and physiology of bone (ed. G.H. Bourne). 1971, III, pp. 1-76.
Academic Press, New York - London
3. Basset, C.A.L. Pulsing electromagnetic fields:
A new method to modify cell behavior in calcified and noncalcified tissues. Calcif. Tissue Int. 1982, 34, 1-8
4. Basset, C.A.L. & Becker, R.O. Generation of electrical potential by bone and response to mechanical stress.
Science. 1962, 137, 1063-1064
5. Basset, C.A.L., Chokshi, H.R., Hernandez, E., Pawluk, R.J.J. & Strop, M. The effect of pulsing electromagnetic fields on cellular calcium and calcification of non-unions. In: Electrical properties of bone and cartilage. Experimental effects and clinical application (eds. C.T. Brighton, J. Black & S.R. Pollack). 1979, pp. 427-441. Grune & Stratton, New York
6. Brighton, C.T., Adler, S., Black, J., Itada, N. & Friedenberg, Z.B. Cathodic oxygen consumption and electrically induced osteogenesis. Clin. Orthop. 1975, 107, 277-282
7. Brighton, C.T., Black, J., Friedenberg, Z.G., Esterhai, J.L., Day, L.J. & Conolly, J.F. A multicenter study of the treatment of non union with constant direct current. J. Bone and Joint Surg. 1981, 63-A, 2-12

8. Brown, K.L.B. & Cruess, R.L. Bone and cartilage transplantation in orthopaedic surgery. *J. Bone Joint Surg.* 1982, 64-A, 270-279
9. Davidovich, Z. & Shanfeld, J.L. Cyclic AMP levels in alveolar bone of orthodontically treated cats. *Arch. Oral. Biol.* 1975, 20, 567-572
10. Dwyer, A.F. & Wickham, G.G. Direct current stimulation in spinal fusion. *Med. J. Aust.* 1974, 1, 73-74.
11. ElMessiery, M.A. Physical basis for piezoelectricity of bone matrix. *I.E.E. Proc.* 1981, 128, 336-346
12. Friedenbergl, Z.B., Andrews, E.T., Smolenski, B.I., Pearl, W.P. & Brighton, C.T. Bone reaction to varying amounts of direct current. *Surg. Gynec. Obstet.* 1970, 131, 894-899.
13. Friedenbergl, Z.B., Roberts, P.G., Didizian, N.H. & Brighton, C.T. Stimulation of fracture healing by direct current in the rabbit fibula. *J. Bone Joint Surg.* 1971, 53-A, 1400-1408
14. Herbst, E. Electric stimulation of bone growth and repair: A review of different stimulation methods. In: *Electric stimulation of bone growth and repair.* (eds. F. Burny, E. Herbst & M. Hinsenkamp). 1978, pp. 1-13. Springer-Verlag, Berlin Heidelberg
15. Herbst, E., Lindskog, S. & Hammarström L. A method for electrical stimulation in vitro. Technical Report 3:81. *Res. Lab. Med. Electr.* 1981. Chalmers University of Technology, Göteborg, Sweden.

16. Herbst, E. & von Satzger, G. Electrical pulsed current stimulation in five cases of congenital pseudoarthrosis of the tibia. In: Electrical properties of bone and cartilage. (eds. C.T. Brighton, J. Black & S.R. Pollack). 1979, pp. 639-664. Grune & Stratton, New York
17. Justus, R. & Luft, J.H. A mechanochemical hypothesis for bone remodelling induced by mechanical stress. *Calc. Tiss. Res.* 1970, 5, 222-235
18. Paterson, D.C., Lewis, G.N. & Cass, C.A. Treatment of delayed union and nonunion with an implanted direct current stimulator. *Clin. Orthop.* 1980, 148, 117-128
19. Petersson, C., Holmer, N.G. & Johnell, O. The effect of transistor-regulated direct current on non-fractured rabbit femur. *Acta Orthop. Scand.* 1982, 53, 161-165
20. Richez, J., Chamay, A. & Bièler, L. Bone changes due to pulses of direct electric microcurrent. *Virchows Arch.* 1972, 357, 11-18
21. Rodan, G.A., Bourret, L.A., Harvey, A. & Mensi, G. 3', 5' cyclic AMP and 3', 5' cyclic GMP: mediators of the mechanical effects on bone remodelling. *Science* 1975, 189, 467-469
22. Rodan, G.A., Bourret, L.A. & Norton, I.A. DNA synthesis in cartilage cells is stimulated by oscillating electric fields. *Science* 1978, 199, 690-692
23. Rohlin, M. & Hammarström, L. Whole-body autoradiography of $^{99}\text{Tc}^{\text{m}}$ -labelled pyrophosphate and related compounds in young rats. *Acta Radiol. (Ther.)*. 1976, 15, 71-80

24. von Satzger, G. & Herbst, E. Surgical and electrical methods in the treatment of congenital and posttraumatic pseudoarthrosis of the tibia. Clin. Orthop. 1981, 161, 82-104
25. Shamos, M.H. & Lavine, L.S. Physical bases for bio-electric effects in mineralized tissues. Clin. Orthop. 1954, 35, 177-188
26. Spadaro, J.A. Electrically stimulated bone growth in animals and man. Clin. Orthop. 1977, 122, 325-332
27. Spadaro, J.A. Electrical osteogenesis - role of the electrode material. In: Electrical properties of bone and cartilage. Experimental effects and clinical applications (eds. C.T. Brighton, J. Black & S.R. Pollack). 1979, pp. 189-197. Grune & Stratton, New York.
28. Spadaro, J.A. & Becker, R.O. Function of implanted cathodes in electro-induced bone growth. Med. Biol. Eng. and Comput. 1979, 17, 769-775
29. Trumbore, D.C., Heideger, W.J. & Beach, K.W. Electrical potential difference across bone membrane. Calcif. Tissue Int. 1980, 32, 159-168
30. Ullberg, S. Studies on the distribution and fate of S^{35} -labelled benzyl-penicillin in the body. Acta Radiol. (Suppl.) 1954, No. 116
31. Urist, M.R. Substratum for bone morphogenesis. Dev. Biol. (Suppl.) 1970, 4: 125-163
32. Wittbjer, J., Nosslin, B., Palmer, B. & Thorngren, K-G. Bone formation of transplanted autologous bone matrix in rabbits evaluated by technetium radionuclide bone imaging. Scand J. Plast. Reconstr. Surg. 1982, 16, 23-28

33. Wittbjer, J., Palmer, B., Rohlin, M. & Thorngren, K-G.
Osteogenetic activity in composite grafts of demineralized compact bone and marrow. Clin. Orthop. 1983, 173, 255-264
34. Wittbjer, J., Palmer, B. & Thorngren, K-G. Osteogenetic properties of reimplanted decalcified and undecalcified autologous bone in the rabbit radius. Scand. J. Plast. Reconstr. Surg. 1982, 16, 239-244
35. Yasuda, Y. Fundamental aspects of fracture treatment. J. Kyoto Med. Soc. 1953, 4, 395-406. (Translated in Clin. Orthop. 1977, 124, 5-89)

